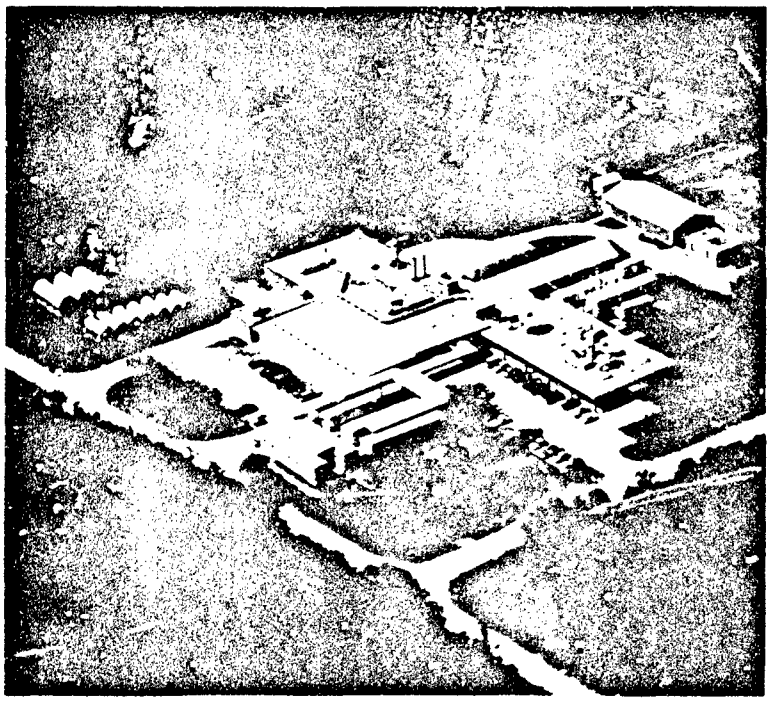
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FINAL REPORT

Task 89-02:

Investigation of an Atropine Radioimmunoassay

To

U.S. Army Medical Research and Development Command

November, 1992

92-32868

Contract DAMD17-89-C-9050

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The purpose of this task was to produce and characterize rabbit anti-atropine antisera for future analytical studies, and to establish and validate the Walter Reed Army Institute of Research (WRAIR) atropine radioimmunoassay (RIA) at Battelle's Columbus Laboratories. For Phase I of this task, an atropine-BSA conjugate was prepared and characterized. This conjugate had an atropine:BSA molar ratio of approximately 32:1, and was used to immunize 12 atropinase-free New Zealand White rabbits for periods of 13 or 21 months using two separate schedules. Antisera were collected and titrated using the RIA procedure. Two rabbits having the highest titer were identified, and their sera were pooled for RIA development. Although the titers of these sera were 10-20-fold lower than antisera from Dr. Smallridge at WRAIR, the RIA characteristics were similar. Using a 1:600 dilution, standard curves were generated with consistent detection limits of 3.0 ng/mL and response ranges extending to 20 ng/mL. A representative standard curve had a slope of 0.984, a y-intercept of 5.975, an ED_{50} of 432.7 pg/50 μL, and a bound cpm/total cpm (B/T) value of 0.32.

The pooled antibody was evaluated for specificity with various atropine analogues, and crossreactivity was obtained only with L-hyoscyamine (3.5 percent), and d,l-homatropine (9.4 percent). Scopolamine, acetylcholine iodide, atropine methyl nitrate, tropic acid, and tropine showed no significant crossreactivity (< 1.4 percent). The WRAIR anti-atropine antisera reportedly crossreacts with L-hyoscyamine (2.4 percent) and d,l-homatropine (3.3 percent) at similar levels[1]. Binding constants for the Battelle and WRAIR antisera were determined from Scatchard plots to be 9.75×10^{-11} M^{-1} and 2.8×10^{-10} M^{-1}, respectively.

In Phase II, the atropine RIA was established and validated at Battelle's laboratories using the Standard Operating Procedure and antisera provided by Dr. Smallridge, WRAIR. Comparable response criteria were demonstrated for the assay. Using a 1:1600 dilution of the WRAIR antisera, the atropine RIA had a detection limit of 1.0 to 1.5 ng/mL, a response range extending to 20 ng/mL, and a B/T of 0.60. The accuracy of the method was demonstrated in an interlaboratory comparison. Spiked rhesus monkey serum samples were prepared at WRAIR and were analyzed at Battelle and WRAIR. The correlation coefficient of the interlaboratory comparison was 0.98 with a slope of 0.928 and an intercept of 13.94. The recovery of atropine from spiked samples was within 20 percent of the target value at 0, 150, and 500 pg/50 μL, and within 23 percent of the target value at 50 pg/50 μL.

In summary, the WRAIR atropine RIA was established and validated at Battelle, and additional anti-atropine antisera sufficient for the analysis of more than 24,500 samples (205 mL at 1:600 dilution) were prepared and characterized. However, high-titered antisera were not obtained during these studies, possibly due to the chemical instability of atropine and/or the conjugation chemistry.

FINAL REPORT

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TASK 89-04

to

U.S. ARMY MEDICAL RESEARCH
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EXECUTIVE SUMMARY

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TASK 89-02:

INVESTIGATION OF AN ATROPINE RADIOIMMUNOASSAY

1.0 INTRODUCTION

An atropine radioimmunoassay (RIA) developed by Wurzberger at al.[1] has been used routinely by the U.S. Army at the Walter Reed Army Institute of Research (WRAIR). This task was requested by the U.S. Army Medical Research and Development Command (USAMRDC) to produce additional anti-atropine antisera for future studies, and to establish this RIA capability at Battelle Columbus Laboratories.

The task was performed in two phases. In the first phase, rabbit anti-atropine antisera were produced and characterized in order to provide an antibody source for future pharmacokinetic studies. Atropine sulfate was conjugated to bovine serum albumin (BSA) using a water soluble carbodiimide reaction. Rabbits were immunized with the atropine-BSA conjugate and anti-atropine antibodies were produced. The antibodies were characterized for specificity and binding constant, and were incorporated into an established atropine RIA.

In the second phase, the atropine RIA was established and validated at Battelle's laboratories. Using the Standard Operating Procedure and rabbit anti-atropine antibodies provided by Dr. Robert Smallridge of WRAIR, Battelle developed and validated the atropine RIA procedure. The two phases of the task were conducted concurrently.

2.0 MATERIALS AND METHODS

2.1 Phase I. Preparation of Anti-Atropine Antisera

2.1.1 Preparation of Atropine-BSA Conjugate

An atropine-protein conjugate was prepared as an immunogen using the procedure described by Wurzberger et al.[1] One hundred thirty seven milligrams (1 mmol) of p-aminobenzoic acid (PABA; Sigma Chemical Co., St. Louis, MO) were dissolved in 6 mL ice-cold 0.2 N HCl by stirring in an ice water bath (reaction vessel number 1). Ninety milligrams (1.3 mmol) of $NaNO_2$

(Sigma Chemical Co., St. Louis, MO) was dissolved in ice cold deionized water and was added dropwise with stirring. Four milliliters of ice-cold, 50 percent dimethylformamide (DMF; Sigma Chemical Co., St. Louis, MO) was added and the reaction was allowed to proceed for 50 min at 0 degrees to 2 degrees C. Twenty nine milligrams (0.3 mmol) of sulfamic acid (Sigma Chemical Co., St. Louis, MO) was dissolved in ice cold deionized water and added at the conclusion of the incubation to quench the unused $NaNO_2$.

Seven hundred seventy eight milligrams (1.33 mmol) of atropine sulfate (Sigma Chemical Co., St. Louis, MO) were dissolved in 20 mL 0.1 M sodium borate, pH 9.0. The atropine solution was then stirred on ice (reaction vessel number 2) as diazotized PABA from reaction vessel number 1 was added dropwise. The reactants were then stirred at 0 degrees to 2 degrees C in the dark. The pH in reaction vessel number 2 was adjusted to 9.0 with 1 N NaOH, and the mixture was stirred overnight at 0 degrees to 2 degrees C in the dark.

The diazo reaction mixture (vessel number 2) was then brought to room temperature. At this stage, the reaction mixture was dark red. Two milliliters of buffer (0.2 M NaH_2PO_4, 0.2 percent NaN_3, pH 5.5) were added to the reaction and the pH was adjusted to 6.0 with 1.0 N HCl. The total volume of the reaction mixture was approximately 40 mL.

Forty four milligrams of N-hydroxysulfosuccinimide (sulfo-NHS; Pierce, Rockford, IL) and 800 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; Pierce, Rockford, IL) were added simultaneously to reaction vessel number 2, giving final concentrations of 5 mM and 100 mM, respectively. Two hundred milligrams (2.98 μmoles) of bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) were dissolved in 10 mL of 1 M KH_2PO_4 and, 0.1 percent NaN_3, at a pH of 8.0, and the BSA solution was added to reaction vessel number 2. The pH of the mixture was adjusted to 8.0 and the mixture was stirred 1 hr at room temperature. The reaction vessel was transferred to a refrigerator at 4 degrees C and stirred overnight.

The contents of reaction vessel number 2 were transferred to pre-boiled dialysis tubing (12,000 MW cutoff) and were dialyzed at 4 degrees C for 6 hr against 2 x 1 L of 20 percent DMF. The conjugate was further dialyzed at 4 degrees C against 6 x 3 L of 0.15 M NaCl over a 3-day period. The dialyzed atropine-BSA conjugate was filtered through Whatman number 1 filter paper.

The volume was adjusted to 200 mL (1 mg atropine-BSA/mL) with the diffusate from the last change of dialysis buffer. The conjugate was aliquoted and stored at ≤ -70 degrees C.

2.1.2 Determination of Free Amino Groups

The concentration of free amino groups and the level of atropine substitution on BSA were determined using the 2,4,6 trinitrobenzene-sulfonic acid (TNBS; Sigma Chemical Co., St. Louis, MO) method[2].

The TNBS reaction was performed as illustrated in the following table. Each sample determination was run in duplicate. The contents of each tube were mixed and incubated at 40 degrees C for 30 min. The reaction was stopped by the addition of 100 µL of 1 N HCl. The absorbance at 335 nm (A_{335}) was determined against an appropriate blank using a D4-50 spectrophotometer. A molar extinction coefficient ($\epsilon_{335\ nm}$) of 10,000 was used to determine the concentration of free amino groups.

Tube #	Sample	Volume of Each Component to be Added			
		Sample (µL)	Saline (µL)	4% $NaHCO_3$ (µL)	0.1% TNBS (µL)
1	Blank-1	0	560	340	0
2	BSA	50	510	340	0
3	BSA	100	460	340	0
4	Atr-BSA	50	510	340	0
5	Atr-BSA	100	460	340	0
6	Blank-2	0	230	340	330
7	BSA	50	180	340	330
8	BSA	100	130	340	330
9	Atr-BSA	50	180	340	330
10	Atr-BSA	100	130	340	330

2.1.3 UV Spectroscopy

Absorption spectra of BSA (0.1 mg/mL), atropine sulfate (1.0 mg/mL), and atropine-BSA conjugate (0.1 mg/mL) were determined using a Beckman DU-50 spectrophotometer.

2.1.4 Size Exclusion Chromatography

BSA and the atropine-BSA conjugate were analyzed using size exclusion chromatography. Samples of each in 50 mM K_2HPO_4 and 150 mM NaCl at pH 7.0 were analyzed on a Superose-6 column (Pharmacia, Piscataway, NJ) using an FPLC system (Pharmacia, Piscataway, NJ) at a flow rate of 1.0 mL/min.

2.1.5 Antisera Production

Anti-atropine antibodies were produced in atropinase-free, male New Zealand White rabbits. The rabbits were screened for atropinase activity using Medical Research and Evaluation Facility (MREF) Standard Operating Procedure (SOP-88-48) prior to beginning the immunization schedule. Two immunization schedules were employed. In the first schedule, four rabbits were immunized with atropine-BSA conjugate prepared in monophosphoryl lipid A with trehalose dicorynomycolate (MPL + TDM) adjuvant (Ribi Immunochem, Hamilton, MT). Animals were immunized for 21 months. Initially, and at the conclusion of the study, rabbits were re-immunized at three-week intervals. From month two through month 19, rabbits were immunized at two-month intervals. Rabbits were immunized with 1 mL of the adjuvant-antigen emulsion containing 200 μg atropine-BSA. For the first two months of the study, the injections were distributed as follows: 300 μL intradermally (ID; 50 μL/site x 6 sites on back), 400 μL intramuscularly (IM; 200 μL/hind leg), 100 μL subcutaneously (SQ) at one site on the neck, and 200 μL intraperitoneally (IP). During months 4 through 7, animals received 40 ID injections on the back with 25 μL/injection site. For the last 12 months of the study, rabbits were immunized with 500 μL IM (250 μL/hind leg) and 500 μL SC (2 x 250 μL/site in neck). Sera were obtained from animals ten days after each boost.

An additional eight rabbits were immunized according to a second schedule. In this regimen, animals were injected with an emulsion containing 500 μg atropine-BSA in Freund's complete adjuvant (GIBCO, Grand Island, N.Y.) for the initial immunization and, 250 μg atropine-BSA in Freund's incomplete adjuvant thereafter. All injections were performed in a volume of 3 mL distributed as follows: 1 mL IM (0.5 mL/hind leg), 1 mL SC (2 x 0.5 mL in the subscapular region) and 1.0 mL ID (10 x 0.1 mL on the back). Serum samples were obtained 10 days after each re-immunization.

Anti-atropine antibody titers were monitored regularly by determining percent bound cpm/total cpm (B/T) for serially diluted sera from 1:100 to 1:12,800. The RIA was performed according to the Standard Operating Procedure prepared by Battelle (Appendix B). Day 1 addition of sera and reagents to RIA tubes was performed with a Tecan RSP 5052 Robotic Sample Processor (Tecan U.S., Hillsborough, NC). The assay specificity of the pooled anti-atropine antisera was evaluated using atropine structural analogs and atropine metabolites. l-hyoscyamine hydrochloride, atropine methyl nitrate, (-) scopolamine hydrochloride, d,l-homatropine hydrobromide, acetylcholine iodide, tropine, and d,l-tropic acid were purchased from Sigma Chemical Co., St. Louis, MO. These compounds and atropine sulfate were prepared in 10 mM Na_2HPO_4 containing 150 mM NaCl, at a pH of 7.5 to give final concentrations of 0.148, 1.48, 14.8, and 148 nM in the RIA. The compounds were evaluated in the atropine RIA and percent bound cpm/background cpm (B/Bo) was determined at each concentration.

The antibody binding constant was determined using Scatchard plots. Independent determinations were made using data from two atropine standard curves and the values were averaged. Scatchard plots were produced using Riacalc™ software, v. 2.65 (Pharmacia LKB Inc., Gaithersburg, MD) by plotting B/F versus nM atropine.

2.2 Phase II. Establish and Validate Atropine RIA at Battelle

Battelle was provided with a Standard Operating Procedure for the atropine RIA (Appendix A) and with rabbit anti-atropine antisera by Dr. Robert Smallridge of WRAIR. Battelle established this method by optimizing the assay and performing validation experiments. The RIA was performed manually according to the SOP. Battelle performed standard curves to demonstrate the detection limit and the response range. The precision of the standard curve was investigated by performing from 2 to 14 replicates of each atropine standard.

Rhesus monkey serum samples were spiked with atropine sulfate at 2, 10, and 20 ng/mL. Battelle analyzed these samples to demonstrate the accuracy of this procedure. Battelle and WRAIR each prepared Rhesus monkey serum samples spiked with atropine at 0, 50, 150, and 500 pg/50 µL. Aliquots of spiked samples were exchanged between labs and each lab analyzed all spiked samples.

The purpose of this interlaboratory comparison was to demonstrate the accuracy of Battelle's method and the equivalency of results between WRAIR and Battelle.

2.2.1 Assay Automation

The atropine RIA was converted to a semi-automated procedure due to the many steps and labor intensive nature of this method (see Atropine RIA SOP in Appendix B). The addition of assay samples and reagents to RIA tubes on day 1 (standards, samples, buffer, normal serum, anti-atropine antibody, and ^{3}H-atropine) was performed using a Tecan RSP Model 5052 Robotic Sample Processor (Tecan U.S., Hillsborough, NC). The system was controlled using an IBM Model 55SX computer and the Application Method Integrator (AMI) Software, v. 7.31 (Tecan U.S., Hillsborough, NC). On day 2, addition of saturated ammonium sulfate, 50 percent saturated ammonium sulfate solution, distilled water, and Hydrofluor (National Diagnostics Inc., Manville, NJ) was performed manually. The assays used in antibody production including titrations and specificity testing were performed using the semi-automated procedure.

3.0 RESULTS

3.1 Phase I. Preparation of Anti-Atropine Antisera

3.1.1 Preparation of Atropine-BSA Conjugate

Atropine is an organic compound which requires coupling to a larger carrier protein to elicit antibody formation. It was conjugated to bovine serum albumin (BSA) through a heterobifunctional crosslinker, p-aminobenzoic acid (PABA), according to the following scheme. In the first reaction, PABA was activated with nitric and hydrochloric acid to yield a diazonium salt. Next, a carboxyl group was introduced into the atropine structure by reacting diazotized PABA with atropine sulfate. Finally, the carboxylic group of the newly synthesized azo derivative of atropine was coupled to the ϵ-amino groups

of BSA by a water-soluble carbodiimide method. Uncoupled reactants and by-products were removed by exhaustive dialysis.

3.1.2 UV Absorption Spectroscopy

UV spectral analysis was performed to determine the incorporation of atropine to BSA. UV absorption spectra of BSA, atropine sulfate, and atropine-BSA are shown in Figures 1, 2, and 3, respectively, and summary data are tabulated in Table 1. λ_{max} for atropine occur at 250 nm, 256 nm, and 262 nm and are distinct from BSA (278 nm). The atropine-BSA conjugate had a λ_{max} at 268 nm and 340 nm corresponding to the bound atropine and the azo group of the crosslinker, respectively. These results indicate the covalent coupling of atropine onto BSA through the PABA crosslinking reagent.

3.1.3 TNBS Analysis

The TNBS assay was used to quantitate indirectly the incorporation of atropine into BSA by quantitating the remaining free ϵ-amino groups after the coupling reaction. According to the Beer-Lambert law, the concentration (C) of a substance equals its absorbance (A) divided by the molar extinction coefficient (ϵ). For the TNBS reaction, the ϵ for BSA is 10^4. Absorbency values for this assay were converted to molar concentration by calculating $A_{335\ nm}/10^4$ and to nanomoles amino groups/mL by calculating $A_{335} \times 10^2$. The BSA protein content was also determined by absorbance at 280 nm using $E^{0.1\%}_{280nm}$ of 0.66. A BSA solution in saline had an A_{280nm} value of 0.573. Using $E^{0.1\%}_{280\ nm}$ = 0.66 for the BSA, corresponding to a concentration was 0.87 mg protein/mL or 13 nanomoles protein/mL (67,000 molecular size for BSA). The atropine-BSA conjugate was adjusted to 1 mg protein/mL in saline (14.9 nanomoles protein/mL) and then used in the TNBS assay. The average number of free amino groups for BSA is 39.5 (Table 2). The average number of free amino groups in the atropine-BSA conjugate was 7.2. Thus, the estimated atropine:BSA molar ratio was 32:1. In general, a hapten:protein ratio of 10:1 to 100:1 is acceptable as an immunogen for antibody production[3].

3.1.4 Size-Exclusion Chromatography of Atropine-BSA

The molecular size of the atropine-BSA conjugate was determined by size exclusion chromatography. Superose-6 gel filtration chromatographic profiles of BSA and atropine-BSA are shown in Figures 4 and 5, respectively. It can be seen that the conjugate was a mixture of monomeric and oligomeric species of atropine-BSA. Oligomeric species were generated during the coupling of carboxyl derivative of atropine to BSA by the EDC method as a result of intermolecular crosslinking through the carboxyl and amino groups of BSA. A large molar excess (335-fold) of atropine derivative over BSA and pre-activation of the carboxylic groups of the derivative with EDC were used to ensure that the amino groups of BSA reacted mainly with the activated atropine derivative.

3.1.5 Antisera Production and Characterization

Antisera Titer. Twelve New Zealand White rabbits were immunized with atropine-BSA conjugate. Four of these rabbits were immunized with the conjugate prepared in MPL + TDM adjuvant and the remaining eight rabbits were immunized with the conjugate emulsified in Freund's adjuvant. Serum samples from each rabbit were collected and titrated by determining the percent B/T for serially diluted samples. Although each rabbit produced anti-atropine antibody, the percent B/T at a 1:100 dilution varied significantly between animals and ranged from 5 percent to 44 percent (Table 3). None of the preimmunization sera demonstrated significant reactivity with atropine. Of the twelve rabbits, the best responders were rabbit E6345 in Group 1 and rabbit 33088 in Group 2 as measured by percent binding at 1:100 dilution. The development of the anti-atropine responses of rabbits E6345 and 33088 during the immunization period plateaued for both rabbits at 9 months and no significant increases in titer were obtained with additional immunization (Figure 6). A pooled serum sample containing aliquots from later bleeds of rabbits E6345 and 33088 was prepared and used for additional characterization studies and in the atropine RIA.

Optimization of Antisera Dilution in Atropine RIA. Following the initial titration based on percent B/T, the optimal dilution of the pooled sera was determined for the standard curves of the atropine RIA. Anti-atropine sera at 1:100, 1:200, 1:400, and 1:600 dilutions were tested and optimal assay characteristics for sensitivity and response range were obtained with the 1:600 dilution. A representative standard curve for this dilution is presented in Figure 7 and has a slope of 0.984, y-intercept of 5.975, ED_{50} of 432.7 pg/50 μL, B/T value of 0.32, and correlation coefficient of 0.9903. Comparing 5 standard curves, the detection limit was variable at the 0.5 ng/mL (25 pg/50 μL) and 1.5 ng/mL (75 pg/50 μL) levels but consistent at the 3.0 ng/mL level (150 pg/50 μL). Each of these values is based on quadruplicate determinations for the reference and duplicate determinations for the standard points. Therefore, a detection limit of 3.0 ng/mL was selected. The upper limit of the response range extended uniformly 20 ng/mL (1000 pg/50 μL). Based on a 1:600 dilution and 205 mL of antisera, Battelle has an adequate antisera inventory to analyze approximately 24,500 samples in duplicate with standards and controls as part of this total.

Specificity Studies. The specificity of the pooled anti-atropine antibody was investigated following the optimization of the RIA. The following compounds were evaluated for crossreactivity in the atropine RIA: l-hyoscyamine hydrochloride, atropine methyl nitrate, (-) scopolamine hydrochloride, d,l-homatropine hydrobromide, acetylcholine iodide, tropine, and d,l-tropic acid. Compounds were evaluated at 0.148, 1.48, 14.8, and 148 nM. These concentrations were selected to provide concentrations on the atropine inhibition curve as well as levels 10- and 100-fold higher than the approximate mid-point of this curve. The inhibition curves are shown in Figure 8, and the ID_{50} and percent crossreactivity are summarized in Table 4. Scopolamine, acetylcholine iodide, atropine methyl nitrate, tropine, and tropic acid showed no significant crossreactivity ($ID_{50} \geq 148$ nM) with the atropine antisera at the concentrations tested. The crossreactivity of tropine and tropic acid was ≤ 1.4 percent at 50 percent B/Bo, and is within the target range of ≤ 5 percent as specified in the proposal. l-Hyoscyamine and d,l-homatropine showed significant inhibition in the atropine RIA. The ID_{50} for atropine was determined to be 2.11 nM. Evaluations of specificity

were made at 50 percent B/Bo. The ID_{50}s of l-hyoscyamine and d,l-homatropine were determined to be 60.58 and 22.49 nM, respectively, with the crossreactivities at these levels of 3.5 percent and 9.4 percent, respectively. The crossreactivity of the test compounds was less than 20 percent as specified in the proposal.

Antibody Binding Constant. The binding constant of the pooled sera was determined using Scatchard plots of data from the atropine standard curves. The binding constant was determined from six points on the linear portion of two inhibition curves by plotting bound cpm/free cpm (B/F) versus molar atropine concentration. The mean value was determined to be 9.75 x 10^{-11} M^{-1}.

3.1.6 Comparison to WRAIR Antisera

The pooled sera from Battelle and Dr. Smallridge, WRAIR, were compared for assay characteristics in the atropine RIA. Although the total bound cpm/total cpm (R/T) of Battelle's antisera is significantly lower than that of the WRAIR antisera, the slopes and y-intercepts of the linear regression line are comparable (Figure 9 and Table 5). The ED_{20}, ED_{50}, and ED_{80} values were higher for the Battelle antisera; however the detection limit, response range, and binding constants for these antisera were comparable. In general, the characteristics of the Battelle and WRAIR antisera in the atropine RIA were very similar except for the low titer of the Battelle antisera.

3.2 Phase II. Establish and Validate Atropine RIA at Battelle

3.2.1 Establishment of the Atropine RIA

The atropine RIA was developed at Battelle based on the Standard Operating Procedure and rabbit anti-atropine antisera provided by Dr. Robert Smallridge of WRAIR. From inhibition curves, the optimal dilution of antibody was determined to be 1:1600. At this dilution, the percent binding of five standard curves ranged from 59.8 percent to 60.5 percent. The

precision of the atropine RIA at various calibration points was ≤ 10 percent coefficient of variation (CV) for the range of 30 percent to 70 percent B/Bo and ≤ 20 percent CV at standard curve values < 30 percent and > 70 percent B/Bo (Table 6) meeting the criteria established in the proposal. The percent CV was less than 8 percent over the complete range of the standard curve. Using 20 replicates of the reference and ten replicates of the 25, 50, and 75 pg/50 μL standards in the percent B/Bo of the 25 pg/50 μL (0.934 ± 0.035) and 50 pg/50 μL levels (0.853 ± 0.054) were not consistently significantly different from the reference (1000 ± 0.012). Only 75 pg/50 μL (0.743 ≤ 0.070) was consistently significantly different from the reference and, therefore, was selected as the detection limit. A representative standard curve for this atropine RIA is shown in Figure 10. This standard curve has a slope of 1.220, a y-intercept of 5.939, and a correlation coefficient of 0.99413. The ED_{20}, ED_{50}, and ED_{80} are 404.4, 129.9, and 41.71, respectively.

3.2.2 Interlaboratory Comparison

Rhesus monkey serum samples containing atropine were analyzed using the RIA to determine the accuracy of the method. Serum samples containing 2, 10, or 20 ng atropine/mL were analyzed. The data presented in Table 7 demonstrate that the measured level was within 10 percent of the expected value at each atropine concentration. A second experiment was performed in which Battelle and WRAIR prepared experimental samples consisting of 0, 50, 150, and 500 pg atropine/50 μL rhesus monkey serum. The samples prepared at each lab were aliquoted into equal volumes and each laboratory analyzed all of the samples in an interlaboratory comparison. The results of the interlaboratory comparison of samples are illustrated in Figures 11 and 12. For the 30 samples prepared by WRAIR, the correlation coefficient of the interlaboratory comparison was 0.98 with a slope of 0.928 and intercept of 13.94. For the 30 samples prepared at Battelle, the correlation coefficient of the interlaboratory comparison was 0.921 with slope and y-intercept equal to 0.916 and -6.171, respectively. A comparison of the expected values and the values as determined in Battelle's analyses of the WRAIR samples is reported in Table 8. The determined value was within 20 percent of the target value at 0, 150, and 500 pg/50 μL levels (0 percent, 14 percent, and 16 percent, respectively) and was within 23 percent of the target value at

50 pg/50 μL. Based on these studies, the RIA data from WRAIR and Battelle were judged to be equivalent, and approval was obtained from Dr. Smallridge, WRAIR, to use this method for sample analysis.

3.2.3 Assay Automation

The atropine RIA is a multi-step, labor intensive method. It consists of set up, assay procedure, liquid scintillation counting, and data analysis (Table 9). During the assay, critical pipetting steps that could affect assay precision and accuracy were transferred to a Tecan Model 5052 liquid handling system. This system was used to dispense standards, controls, samples, buffer, normal serum, atropine antibody, and ^{3}H-atropine to the reaction tube.

The manual and automated procedures were compared to demonstrate their equivalency before using the automated method for sample analysis or antibody characterization studies in Phase I. The standard curve for the atropine RIA was performed by the manual and automated methods and the data compared. The standard curves from both methods had similar characteristics (Figure 13 and Table 10) but there was less variation with the automated method. The precision of the automated method was found to be equivalent to the manual method with the percent CV values ranging from 1.01 percent to 5.05 percent for the automated procedure and 0.24 percent to 7.63 percent for the manual method (Table 11). In addition, the use of automation reduced the total attended assay time per run from 1240 min (20.67 hr) to 945 min (15.75 hr) and increased the number of samples per day from 57 to 120 by permitting two runs per day. In addition, the assay repeats due to technical errors were reduced from 9.9 percent for the manual method to 3.8 percent with automation. Thus, the benefits of assay automation were increased sample throughput and reduction of assay repeats with assay characteristics equivalent to the manual method.

4.0 CONCLUSIONS

Low molecular weight compounds such as atropine generally require chemical linkage to a much larger carrier molecule in order to facilitate antibody production. In this task, atropine sulfate was linked to a relatively large carrier protein molecule, bovine serum albumin, through atropine's phenolic ring. Attachment at this site has been shown by Fasth et al.[4] and Inoue et al.[5] to produce higher titered antisera than by attachment at other sites such as the tertiary amine of the tropine ring or the hydroxy methyl group of the tropic acid moiety. Antibody production was further enhanced by presenting the conjugate to the rabbits in an emulsion of atropine-BSA conjugate and adjuvant. The adjuvants provide a nonspecific stimulus to the animal's immune system and provide for a prolonged release of the antigen within the animal's body.

The magnitude of individual animal antibody responses to antigens varied significantly which may be attributed to genetic differences. Of the two best responders, one was immunized with antigen in MPL + TDM adjuvant and the other with antigen in Freund's adjuvant. The MPL + TDM animal responded somewhat more slowly, but this may reflect differences in the frequency of immunizations, route of administration, or other factors. The maximum antibody level and duration of the responses was approximately equal for animals receiving each adjuvant. Once the antibody response plateau was achieved, the antibody titer did not increase with continued booster immunizations.

Another explanation for the low antibody titer is the lability of the atropine molecule. Atropine is an ester of tropine and tropic acid. During antibody formation, the atropine-BSA conjugate is phagocytized by macrophages to form intracellular endosomes. These subcellular organelles have an internal pH of 4-5. In addition, during antigenic processing, endosomes fuse with lysosomes containing various degradative enzymes including esterases[6]. Thus, the low pH and esterase content of the endosomes can result in cleavage of this atropine ester linkage leaving tropic acid bound to the BSA carrier protein and releasing free tropine. Therefore, the low antibody titer of these animals may be attributed to the labile nature of atropine conjugated to the BSA.

An antibody pool was created with sera from the two best anti-atropine antibody producing rabbits. The optimal dilution of the antibody was 1:600 for the Battelle antisera and 1:1600 for the WRAIR antisera, and the percent binding at these dilutions was 32 percent and 57 percent, respectively. Approximately 205 mL of the pooled antisera was created and at a 1:600 dilution, this volume is sufficient for analyzing of approximately 24,500 samples in duplicate.

The pooled antisera were evaluated for crossreactivity with a group of structurally related compounds. The hydrolysis products of atropine are tropine and tropic acid, but no crossreactivity was obtained with either compound at concentrations ranging from 0.148 to 148 nM. These results are consistent with findings of Wurzburger et al.[1] and are significant in that the atropine RIA will measure the atropine parent molecule but not the major metabolic products, tropine and tropic acid. A low level crossreactivity (< 10 percent) was obtained with l-hyoscyamine and d,l-homatropine at the ID_{50} level. Hyoscyamine is an l-isomer of atropine and d,l-homatropine has a hydroxy group in place of the hydroxy methyl on the tropic acid moiety. Although these structural differences are minor, 10-30-fold more of these compounds were needed to obtain binding equivalent to atropine. Similar observations were made by Wurzburger et al. Although these data are of interest in understanding the specificity of the antibody, they are not of significance in the clinical application of the method since neither compound is anticipated to be present in plasma samples. Scopolamine and atropine methyl nitrate are also structurally similar to atropine with modifications to the tropine moiety, although the tropic acid moiety is identical to that in atropine. Neither compound crossreacted significantly with the pooled Battelle antisera. Likewise, acetylcholine iodide did not crossreact. These results suggest that the structural differences in the tropine moiety change the immunoreactivity dramatically.

Performance criteria were established in the proposal for the development and validation of the atropine RIA at Battelle using the WRAIR SOP and antibody. The criteria for detection limit, response range, and the precision of the standard curve were met, thus providing a method with experimentally useful assay characteristics for pharmacokinetic studies. An interlaboratory validation was performed to further validate this method. Analysis of identical samples at Battelle and WRAIR showed excellent

correlation. The detection limit of the atropine RIA using Battelle's pooled antisera was 3 ng/mL with a response range extending to 20 ng/mL. These assay characteristics were comparable to the WRAIR antisera and were consistent with the plasma/serum atropine concentrations anticipated in future pharmacokinetic studies.

In summary, the atropine RIA has been established and validated at Battelle. Additional anti-atropine sera of satisfactory quality and quantity has been produced in preparation for future pharmacokinetic studies. This method has also been converted to a time-saving, semi-automated procedure.

5.0 RECOMMENDATIONS

The anti-atropine antibody generated at Battelle is sufficient for sample analyses by the RIA, even though the titer is significantly lower than the titer of anti-atropine antibody obtained from Dr. Smallridge, WRAIR. The titer could be improved by: (1) conjugation of atropine to an alternative carrier protein such as porcine thyroglobulin to obtain a higher level of atropine incorporation; (2) modification of the conjugation chemistry that avoids extremes of pH may prevent cleavage of the atropine molecule at the ester site during the conjugation reaction; (3) analogues of atropine may retain the necessary immunogenicity but with improved stability within the physiological milieu of the animal; (4) the stability of the atropine molecule in vivo may be enhanced by the use of an esterase inhibitor, such as physostigmine.

6.0 ACKNOWLEDGEMENTS

The names, role in the study, and highest academic degree of principal contributors in this study are presented in the following list.

Name	Title	Degree
David W. Hobson	Principal Investigator MREF Manager	Ph.D
Larry S. Miller	Study Director	Ph.D.
Balwant S. Bhullar	Task Leader Conjugate Preparation and Chemical Characterization	Ph.D.
Victor S. Moore	Task Leader Antisera Production and Assay Development	M.S.

The authors wish to acknowledge Suzanne Kellner, Becky Spahr, and Angelia Doye for their technical assistance in this study and to Julie Teeters, Charlotte Hirst, and Carol Tibbs for the preparation of this report. The authors also wish to acknowledge Dr. Robert Smallridge of Walter Reed Army Institute of Research for many helpful discussions during this project and for providing atropine antisera.

7.0 REFERENCES

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APPENDIX A

STANDARD OPERATING PROCEDURE FOR ATROPINE
RIA AS PROVIDED BY WRAIR

Study Specific SOP
No. G8180-4203-010
September 10, 1987

WALTER REED ARMY INSTITUTE OF RESEARCH
DEPARTMENT OF CLINICAL PHYSIOLOGY
STANDARD OPERATING PROCEDURE

TITLE: Radioimmunoassay (RIA) Procedure for the Measurement of Atropine Sulfate.

SCOPE: This SOP outlines the procedures for assaying atropine in serum.

REFERENCES:

1. Wurzburger RJ, RL Miller, BG Boxenbaum, and S Spector, Radioimmunoassay of atropine in plasma. J Pharmacol Exp Therap 203:435, 1977.

2. Kradjan WA, RC Smallridge, R Davis, and P Verma, Atropine serum concentrations after multiple inhaled doses of atropine sulfate. Clin Pharmacol Therap 38:12, 1985.

3. FDA, Nonclinical Laboratory Studies (Good Laboratory Practice Regulations), Federal Register Vol. 43, No. 247, pp. 60013-60025, 22 Dec. 1978.

PROCEDURES:

I. General:

A. The RIA procedure is based on standard techniques using an antibody raised in a rabbit. Antibody bound drug is separated from unbound atropine by ammonium sulfate precipitation. Isotopic counts are determined in a liquid scintillation system and compared to a series of atropine sulfate standards of known concentrations.

B. Technical support for the procedure is performed by Carolyn E. Custom.

II. Specific:

A. Reagents

1. Atropine sulfate antibody: Prepare a dilution to assure binding of 50-70% (titer of ~ 1:1200-1:2200) i.e. prepare a 1:1600 dilution by adding 10 ul of antibody to 15.99 ml phospho-buffered saline (PBS). The serum antibody is stored undiluted at -20°C in 30 ul aliquots. A fresh dilution is prepared the day of each assay.

2. Radioligand (^{3}H-atropine sulfate, Specific Activity = ~30 Ci/mmol): Diluted initially in buffer such that 20 ul = 4,000 cpm. Stored at -20°C.

3. Buffer: PBS, pH 7.3.

4. Normal human serum (NHS)- used in the standard curve and for spiking quality control (QC) samples for human samples. For animal studies, species specific animal serum is used in place of NHS.

5. Liquid Scintillator: Available commercially as Ultrafluor, Hydrofluor, Aquasol, PCS.

B. Preparation of Standards

1. Frozen stock concentrate: 1.0 mg atropine sulfate dissolved in 20.0 ml PBS = 50 ug/ml.

2. Stock A: 10 ul of frozen stock + 19.99 ml buffer = 0.5 ug/20 ml (25 ng/ml). This is prepared fresh before each assay.

3. Stock B: 100 ul of stock A + 2.4 ml buffer = 2.5 ng/2.5 ml (1.0 ng/ml). This is prepared fresh before each assay.

4. Standard curve concentrations

0 pg = Buffer alone	250 pg = 10 ul of stock A
25 pg = 25 ul of stock B	500 pg = 20 ul of stock A
50 pg = 50 ul of stock B	750 pg = 30 ul of stock A
75 pg = 75 ul of stock B	1000 pg = 40 ul of stock A
100 pg = 100 ul of stock B	
150 pg = 150 ul of stock B	

C. Preparation of Quality Control Sera

1. Stock C: 1.0 ml of stock A (25ng) + 1.5 ml normal human serum = 25 ng/2.5 ml (10 ng/ml). For animal studies, species specific animal serum will be used in place of human serum.

2. Stock D: 200 ul of stock A (5 ng) + 2.3 ml normal human serum = 5 ng/2.5 ml (2 ng/ml)

3. QC Concentrations

500 pg = 50 ul of stock C
250 pg = 25 ul of stock C
100 pg = 50 ul of stock D

D. RIA Procedure

1. Addition of reagents: Refer to Table 1 for volumes of each reagent added to the standard curve, nonspecific, QC, and unknown sample assay tubes. Reagents are added to 12 x 75 mm RIA tubes in order, from left to right.

2. Total Counts: Add 20 ul of ^{3}H-atropine to 2 glass scintillation vials.

3. Vortex each RIA tube for 5-10 seconds.

4. Incubate RIA tubes for 18-24 hours at 5°C.

E. Ammonium Sulfate Precipitation

1. Add 0.5 ml of 100% saturated $(NH_4)_2SO_4$. Vortex for 5-10 seconds. Incubate for 15 min at 5°C. Centrifuge at 3000 rpm for 20 min. Aspirate supernate by pasteur pipet.

2. Add 1.0 ml of 50% saturated $(NH_4)_2SO_4$. Vortex for 5-10 seconds. Centrifuge at 3000 rpm for 20 min. Aspirate supernate by pasteur pipet.

3. Add 1.0 ml distilled H_2O to dissolve each precipitate, vortex for 5-10 seconds and transfer to glass scintillation vials.

4. Rinse each RIA tube with 2.0 ml of liquid scintillator and transfer to each scintillation vial.

F. Liquid Scintillation

1. Add 8 ml of liquid scintillator to each vial. Cap vials.

2. Count vials in Tracor Analytic Liquid Scintillation System for 10 minutes.

G. Calculations and Statistics

1. Perform computations and statistical analysis of data with Hewlett Packard 9815A Calculator.

2. Multiply by conversion factor to obtain results in ng/ml.

Technician Signature [signature]

Date 13 Feb 87

Supervisor Approval Robert P. Smallwood, QA Mgr

Date 14 Feb. 1987

TABLE 1. ATROPINE SULFATE RADIOIMMUNOASSAY

Tube #	Concentration	Standard	Sample	Buffer	NRS	Ab	^{3}H-Atr
	Standard Curve						
1-2	Nonspecific Binding			430 µl	50 µl		20 µl
3-6	0 Pg			330 µl	50 µl	100 µl	20 µl
7-8	25 Pg Stock B	25 µl		305 µl	50 µl	100 µl	20 µl
9-10	50 Pg Stock B	50 µl		280 µl	50 µl	100 µl	20 µl
11-12	75 Pg Stock B	75 µl		255 µl	50 µl	100 µl	20 µl
13-14	100 Pg Stock B	100 µl		230 µl	50 µl	100 µl	20 µl
15-16	150 Pg Stock B	150 µl		180 µl	50 µl	100 µl	20 µl
17-18	250 Pg Stock A	10 µl		320 µl	50 µl	100 µl	20 µl
19-20	500 Pg Stock A	20 µl		310 µl	50 µl	100 µl	20 µl
21-22	750 Pg Stock A	30 µl		300 µl	50 µl	100 µl	20 µl
23-24	1000 Pg Stock A	40 µl		290 µl	50 µl	100 µl	20 µl
	Quality Control						
25-26	100 Pg Stock D	50 µl		330 µl		100 µl	20 µl
27-28	250 Pg Stock C	25 µl		330 µl	25 µl	100 µl	20 µl
29-30	500 Pg Stock C	50 µl		330 µl		100 µl	20 µl
	Serum Samples						
31-32	Sample 1		50 µl	330 µl		100 µl	20 µl
33-34	Sample 2		50 µl	330 µl		100 µl	20 µl

APPENDIX B

STANDARD OPERATING PROCEDURE FOR ATROPINE RIA AS PREPARED BY BATTELLE

Manual Number:

Battelle SOP Number: TOX-VE-014-00
Effective Date: December 28, 1990
Page 1 of 10

Key Words: ATROPINE, RADIOIMMUNOASSAY, RIA

Standard Operating Procedure (SOP)

THE DETERMINATION OF SERUM ATROPINE SULFATE CONCENTRATIONS BY RADIOIMMUNOASSAY (RIA)

Originated by: Victor [illegible] Date 12-17-90

Approved by: J. M. [illegible] Date 12/20/90
Manager
Toxicology and Pharmacology Department

Approved by: Richard L. Staub Date 12/21/90
Executive Secretary
Quality Council
Health and Environment Group

Reviewed and Registered by QAU: [illegible] Date 12-21-90

Distribution List:

Quality Assurance Unit
SOP Manual

BATTELLE
BATCOPY

Battelle
Health and Environment Group
505 King Avenue
Columbus, Ohio 43201

BATTELLE

I/II. SCOPE/PURPOSE:

The purpose of this Standard Operating Procedure (SOP) is to describe a Radioimmunoassay method employed in the determination of serum atropine sulfate concentrations.

III. REFERENCES:

1. Wurzburger, R. J., Miller, R[illegible] Boxenbaum [illegible] and S. Spector. 1977. Radioimmunoassay of Atropine in Plasma. J Pharm Therap 203: 435.

2. Kradjan, W. A., Smallridge, R[illegible] Davis, [illegible] and [illegible] Verma. 1985. Atropine Serum Concentrations After Multiple Intravenous Doses of Atropine Sulfate. Clin Pharmacol Ther 38: [illegible]

IV. DEFINITIONS: None

V. PROCEDURES:

Preliminary Tasks

A. Preparation of Phosphate Buffered Saline (PBS), pH 7.5

1. Combine the following components to prepare 1 liter PBS (10 mM Na_2HPO_4, 150 mM NaCl, pH 7.5:

Na_2HPO_4	1.420 grams
NaCl	8.766 grams
distilled water	980.0 ml

2. Adjust the pH to 7.5 with 6 N HCl. Bring the volume to 1000 ml with distilled water.

Store PBS at 1-9°C. The PBS is stable for a period of one month from the date of preparation.

B. Preparation of Saturated Ammonium Sulfate

1. Combine the following reagents to prepare 500 ml saturated ammonium sulfate:

$(NH_4)_2SO_4$	257.6 grams
distilled water	500.0 ml

2. Do not adjust pH. Store at 1-9°C. This reagent is stable for a period of one month from date of preparation. Prepare at least 24 hours prior to use.

C. Preparation of 50 percent Saturated Ammonium Sulfate

1. Combined the following reagents to prepare 500 ml of 50 percent saturated ammonium sulfate:

$(NH_4)_2SO_4$	128 grams
distilled water	500 ml

2. Do not adjust pH. Store at 1-9°C. This reagent is stable for a period of one month from date of preparation. Prepare at least 24 hours prior to use.

D. Preparation of ^{3}H-Atropine Stock Solution

1. ^{3}H-Atropine is prepared in PBS, pH 7.5 at a concentration of approximately 4000 CPM/25 µl. This material is aliquoted and stored at -70 (± 5)°C. The labeled atropine is stable for a period of one year.

2. Thaw one aliquot daily. Dispose of the leftover material at the completion of the experiment according to Battelle SOP for disposal of radioactive materials.

E. Preparation of Primary Atropine Stock Solution

1. Prepare 1 mg/µl solution of atropine sulfate in PBS, pH 7.5. Weigh a minimum of 10 mg atropine sulfate. Mix thoroughly and aliquot. Store at -70 (± 5)°C. The material is stable for a period of one year from the date of preparation.

F. Preparation of Rabbit Anti-Atropine Antisera Stock

1. The correct concentration of rabbit anti-atropine antisera will be determined in preliminary testing. The stock antisera is stored as 30 µl aliquots at -70 (± 5)°C. Dilute the antisera to the proper concentration in PBS, pH 7.5. Prepare the diluted antibody fresh daily. Leftover material may be frozen and used for repeat analyses performed within a period of five days. Thereafter, dispose of the diluted material.

G. Normal Serum

1. A stock of normal serum obtained from the same species as that of the serum samples being analyzed will be aliquoted and

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stored at -70 (± 5)°C. The frozen stock is stable for a period of one year.

2. Aliquot(s) of normal serum are thawed freshly on the assay day. The serum is used undiluted in the assay. Unused material may be frozen and used on a subsequent test day.

H. Test Samples

1. Test samples are stored at -70 (± 5)°C.

RIA Set Up (Day 1)

1. Prepare atropine sulfate Stocks A and B fresh daily from a freshly thawed aliquot of the Primary Atropine Stock solution as follows:

 a) Combine 10 µl Primary Atropine Stock + 990 µl PBS (Dilution a)
 b) Combine 10 µl Dilution a + 990 µl PBS (Dilution b)
 c) Combine 250 µl Dilution b + 750 µl PBS (Stock A)
 d) Combine 10 µl Dilution b + 990 µl PBS (Stock B)

2. Dispose of the leftover Primary Atropine Stock as well as leftover Atropine Stocks A and B and Dilutions a and b at the conclusion of the RIA set up.

3. Prepare Stock C by combining 140 µl Stock A with 1.5 ml of normal sera derived from the same species as the sera under analyses. The volumes may be modified proportionately in order to produce the correct volumes for larger or smaller experiments. Dispose of the unused material at the end of the day.

4. Prepare Stock D by combining 280 µl Stock A with 2.3 ml normal sera derived from the same species as the sera under analyses. The volumes may be modified proportionately in order to prepare the correct volumes for larger or smaller experiments. Dispose of the unused material at the end of the test day.

5. The RIA procedure is set up as described on the attached form entitled "Atropine Sulfate Radioimmunoassay Tube Setup". Reagents are aliquoted to 12 x 75 mm polystyrene RIA tubes in order from left to right as indicated in this form.

6. Upon adding all reagents, vortex each tube 5-10 seconds.

7. Incubate the tubes 20 (± 1) hours at 1-9°C.

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8. Prepare the total counts control by adding 20 µl ^{3}H-atropine to each of two 20 ml scintillation vials. Add 10.0 ml Hydrofluor and 1.0 ml distilled water to each vial and mix.

Completion of RIA (Day 2)

1. Add 0.5 ml 100 percent saturated ammonium sulfate to each RIA tube. Vortex for 5-10 seconds. Incubate for 30 minutes at 1-9°C. Centrifuge at approximately 2800 RPM (1550 x g) for 30 minutes at room temperature (RT). Carefully aspirate the supernate with a pasteur pipet and transfer the liquid to a container for radioactive liquid waste.

2. Add 1.0 ml 50 percent saturated ammonium sulfate to each tube. Vortex for 5-10 seconds. Centrifuge at approximately 2800 RPM (1550 x g) for 30 minutes at RT. Aspirate the supernate with a pasteur pipet and transfer to a container for radioactive liquid waste.

3. Add 1.0 ml distilled water to each tube to dissolve the pellet. Vortex for 5-10 seconds.

4. Transfer the contents of each RIA tube to a separate scintillation vial by careful pouring. Rinse the RIA tubes with 2.0 ml Hydrofluor and transfer the rinse to the respective vial.

5. Add 10.0 ml Hydrofluor to each scintillation vial and mix.

6. Count the vials for 10 minutes or to a preset error of 0.5 percent on a liquid scintillation counter.

Data Analysis

Data analysis is performed using RiaCalc DM, Version 2.65 (Pharmacia Wallac). Data is reported as ng/ml.

VI. QUALITY CONTROL

1. All equipment and instruments will be operated, calibrated, and maintained according to their respective SOPs.

2. The study director or his designee will review all raw data, completed data forms and other pertinent study records.

BASF

3. The form entitled "Atropine Sulfate Radioimmunoassay Tube Setup" details the contents of each standard, control, and sample tube and will be employed daily during assay set up to ensure correct distribution of reagents.

4. The form entitled "Record For Instruments, Equipment, Reagents Used For Radioimmunoassay" will be used to document all reagents and equipment used in an assay.

5. The form entitled "Atropine Sulfate RIA Run Sheet" will be utilized to record the identification, the assay sequence for controls and samples for an assay.

6. Preparation of buffers and other reagents will be recorded on the attached form entitled "Buffer/Reagent Preparation."

7. A series of low, medium, and high controls are included in each experiment to assess the quality of each experiment. Control data will be tabulated for each run and will be reviewed by the study director.

8. Additional control parameters such as the slope, the y-axis and intercept of the regression curve, and other parameters are computed by RiaCalc-PC. These will be tabulated for each experiment and reviewed by the study director.

BATTELLE

BUFFER/REAGENT PREPARATION

Study: _____

Project: _____ Date: _____

Buffer/Reagent: _____

Buffer Storage Conditions: _____ Buffer Expiration Date: _____

Constituents:

Reagent	Supplier	Lot	Receipt Date	Expiration Date	Amount

Balance: Description: _____

Balance ID: _____ Last Calibration: _____

Standard Weights: Balance ID: _____

Determination No.	Actual Wt.	Wt. Read
1		
2		
3		
4		

pH Adjustment (Reagent and Volume): _____

pH Meter/ISCD ID: _____ Final pH: _____

Comments:

Prepared By: _____ Date: _____

Reviewed By: _____ Date: _____

ARCHIVAL COPY

ATROPINE SULFATE RADIOIMMUNOASSAY TUBE SETUP

STUDY CONTROL No._____ PROJECT No.:_____

DATE:_____ RUN No._____ PAGE No:_____

Tube No.	Conc.	Standard	Sample	Buffer	Normal Serum	Antibody	^{125}I-Atropine Sulphate
	Standard Curve						
1	T. Tube	None					20 uL
2	T. Tube	None					20 uL
3	NSB	None			50 uL		20 uL
4	NSB	None			50 uL		20 uL
5	0 pg	None		330 uL	50 uL	100 uL	20 uL
6	0 pg	None		330 uL	50 uL	100 uL	20 uL
7	0 pg	None		330 uL	50 uL	100 uL	20 uL
8	0 pg	None		330 uL	50 uL	100 uL	20 uL
9	25 pg	25 uL Stock C		305 uL	50 uL	100 uL	20 uL
10	25 pg	25 uL Stock C		305 uL	50 uL	100 uL	20 uL
11	50 pg	50 uL Stock B		280 uL	50 uL	100 uL	20 uL
12	50 pg	50 uL Stock B		280 uL	50 uL	100 uL	20 uL
13	75 pg	75 uL Stock B		255 uL	50 uL	100 uL	20 uL
14	75 pg	75 uL Stock B		255 uL	50 uL	100 uL	20 uL
15	100 pg	100 uL Stock B		230 uL	50 uL	100 uL	20 uL
16	100 pg	100 uL Stock B		230 uL	50 uL	100 uL	20 uL
17	150 pg	150 uL Stock B		180 uL	50 uL	100 uL	20 uL
18	250 pg	250 uL Stock B		130 uL	50 uL	100 uL	20 uL
19	250 pg	20 uL Stock A		320 uL		100 uL	20 uL
20	250 pg	20 uL Stock A		320 uL	50 uL	100 uL	20 uL
21	500 pg	20 uL Stock A		310 uL	50 uL	100 uL	20 uL
22	500 pg	20 uL Stock A		310 uL	50 uL	100 uL	20 uL
23	750 pg	30 uL Stock A		300 uL	50 uL	100 uL	20 uL
24	750 pg	30 uL Stock A		300 uL	50 uL	100 uL	20 uL
25	1000 pg	40 uL Stock A		290 uL	50 uL	100 uL	20 uL
26	1000 pg	40 uL Stock A		290 uL	50 uL	100 uL	20 uL
	Quality Control						
27	100 pg	50 uL Stock D		330 uL		100 uL	20 uL
28	100 pg	50 uL Stock D		330 uL		100 uL	20 uL
29	250 pg	25 uL Stock C		330 uL	25 uL	100 uL	20 uL
30	250 pg	25 uL Stock C		330 uL	25 uL	100 uL	20 uL
31	500 pg	50 uL Stock C		330 uL		100 uL	20 uL
32	500 pg	50 uL Stock C		330 uL		100 uL	20 uL
	Samples (Atropine Run List)		50 uL	330 uL		100 uL	20 uL
	Samples (Atropine Run List)		50 uL	330 uL		100 uL	20 uL

Technician Signature: _____ Date: _____

Reviewed By: _____ Date: _____

Atropine Sulfate RIA Run List

(Radioimmunoassay Laboratory)
Battelle, 505 King Avenue, Columbus, OH 43201

Date: _____ Run No._____ Page No._____
Study Control No:_____ Project No._____

Tube No.	Sample ID or Code (Source)	Draw Time	Draw Date	Concentration pg/SUSC [illegible] ng/mL	Comments

Operator Signature: _____ Date: _____
Reviewed By: _____ Date: _____

EXAMPLE

RECORD FOR INSTRUMENTS, EQUIPMENT, REAGENTS USED FOR RADIOIMMUNOASSAY

Project:	Assay:	Project No.
		SC No.

LIST OF INSTRUMENTS/ EQUIPMENT USED

SN	Instrument/ Equipment	Model	Battelle ID	Location
1	Gamma Counter			
2	Scintillation Counter			
3	Water Bath (Temp.)			
4	Heating Blocks/Dry Bath (Temp.)			
5	Incubator (Temp.)			
6	Refrigerator (Temp.)			
7	Freezer (Temp.)			
8				

Other: Incubation Time: [illegible]ne:
Out Ti[illegible]

[illegible]S, CHEMICALS, SOLVENTS AND REAGENTS USED

SN	Name	C[illegible]	Lot No.	Exp. Date
1				
2				
3				
7				
10				
11				
12				

Comments:

Technician Signature:	Date:
Reviewed By:	Date:

EXAMPLE

APPENDIX C

FIGURES

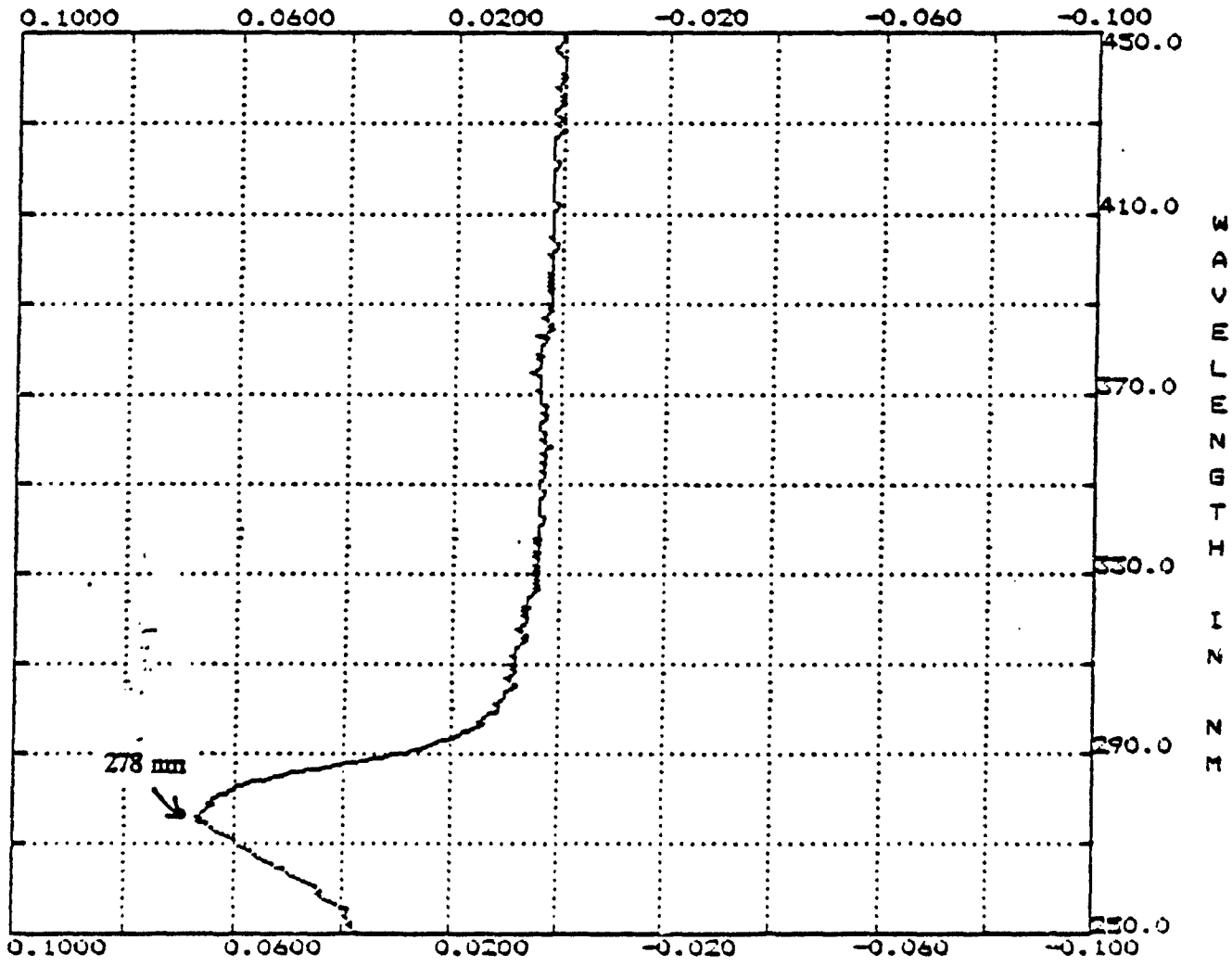


FIGURE 1. ABSORPTION SPECTRUM OF BSA

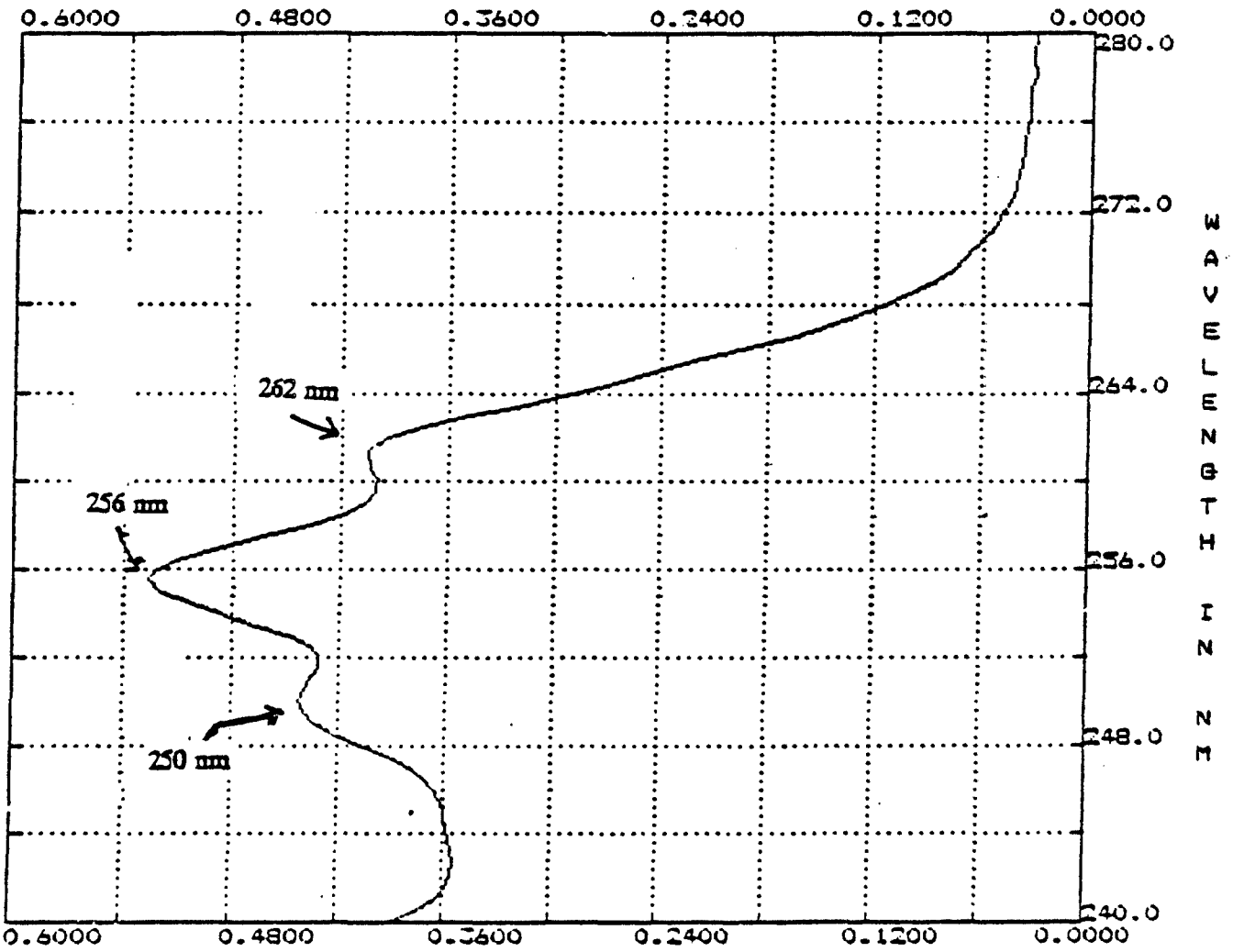


FIGURE 2. ABSORPTION SPECTRUM OF ATROPINE SULFATE

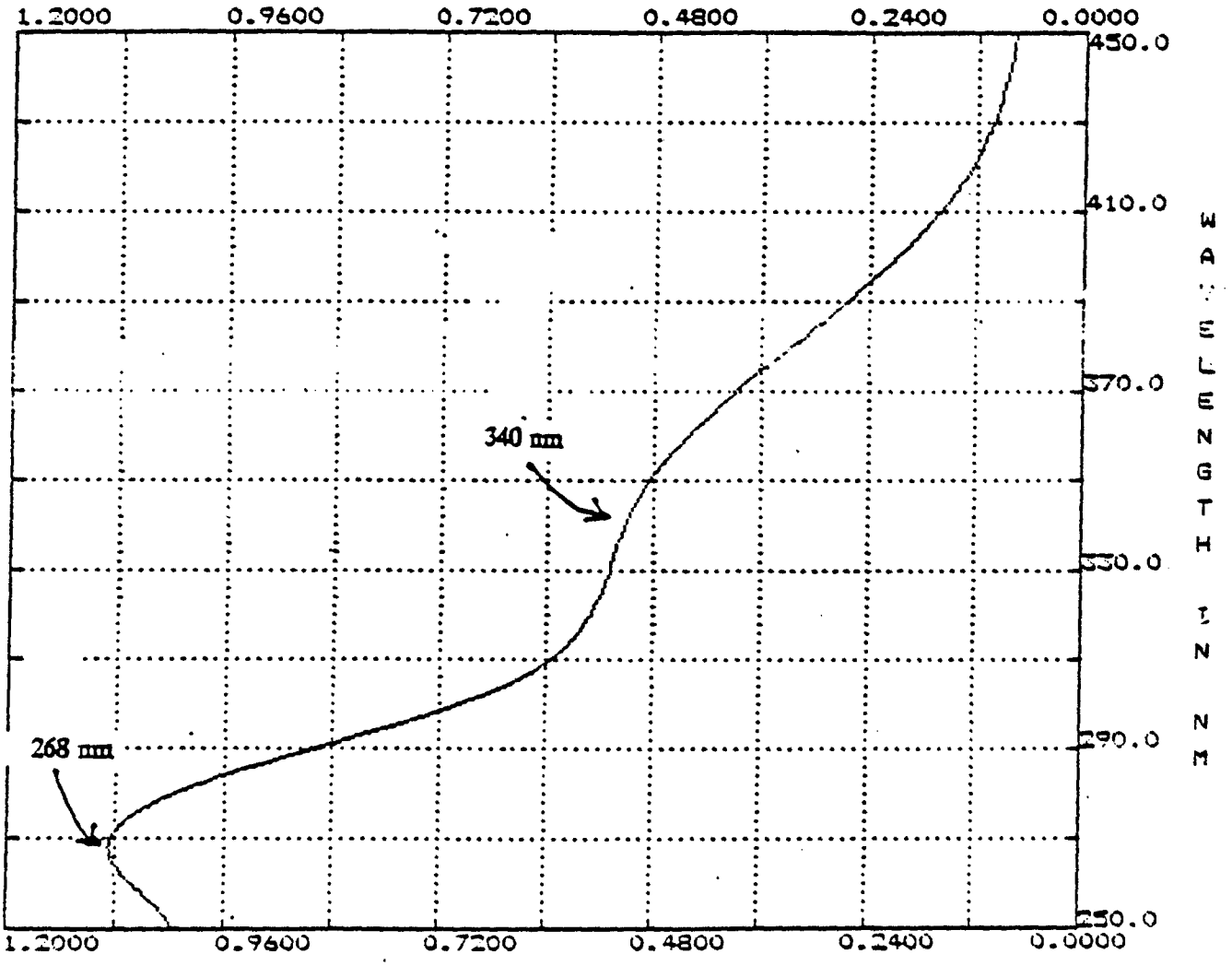


FIGURE 3. ABSORPTION SPECTRUM OF ATROPINE-BSA

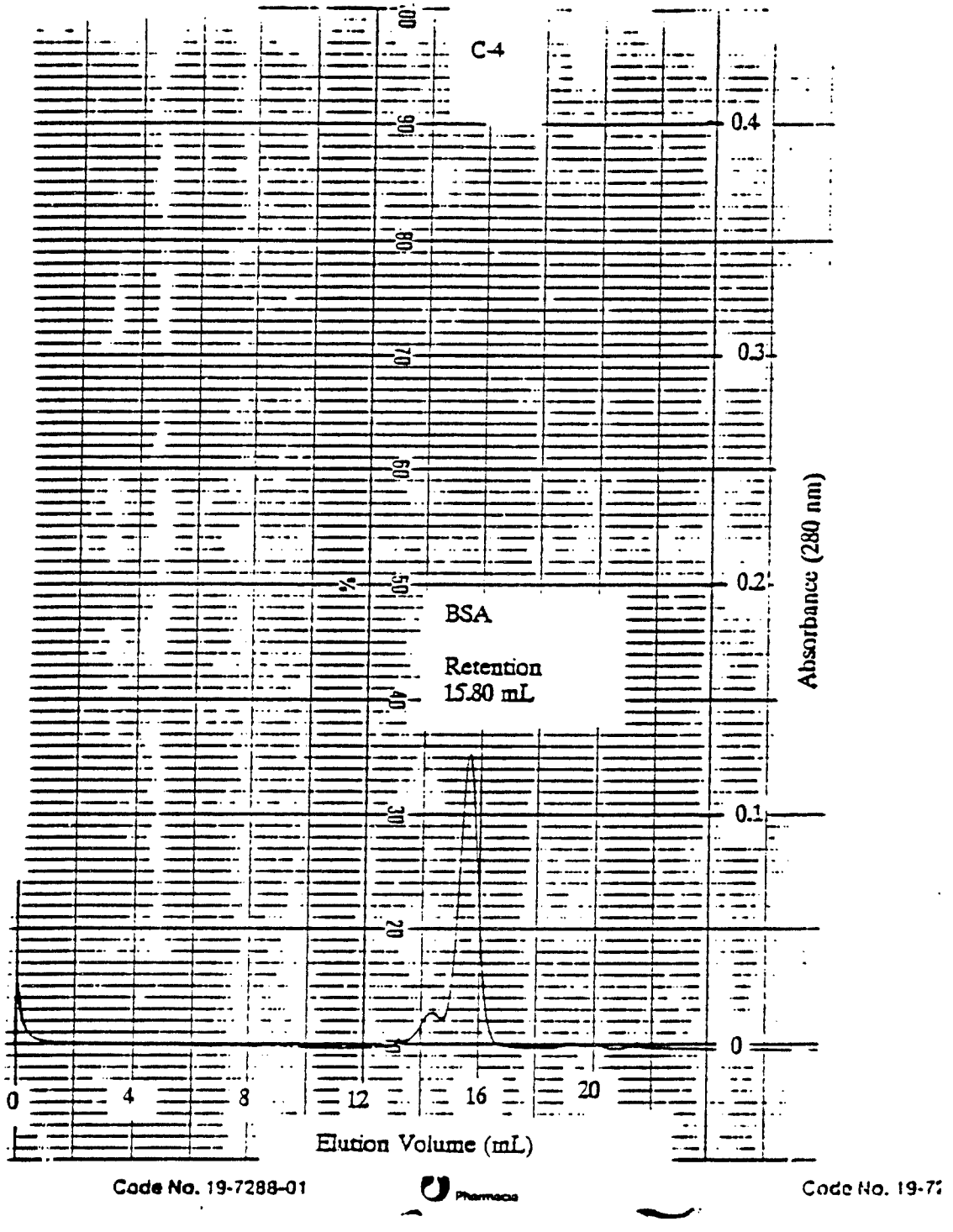


FIGURE 4. SIZE EXCLUSION CHROMATOGRAPHY OF BSA. 200 µL (1 mg/mL) WAS APPLIED TO A SUPEROSE-6 COLUMN

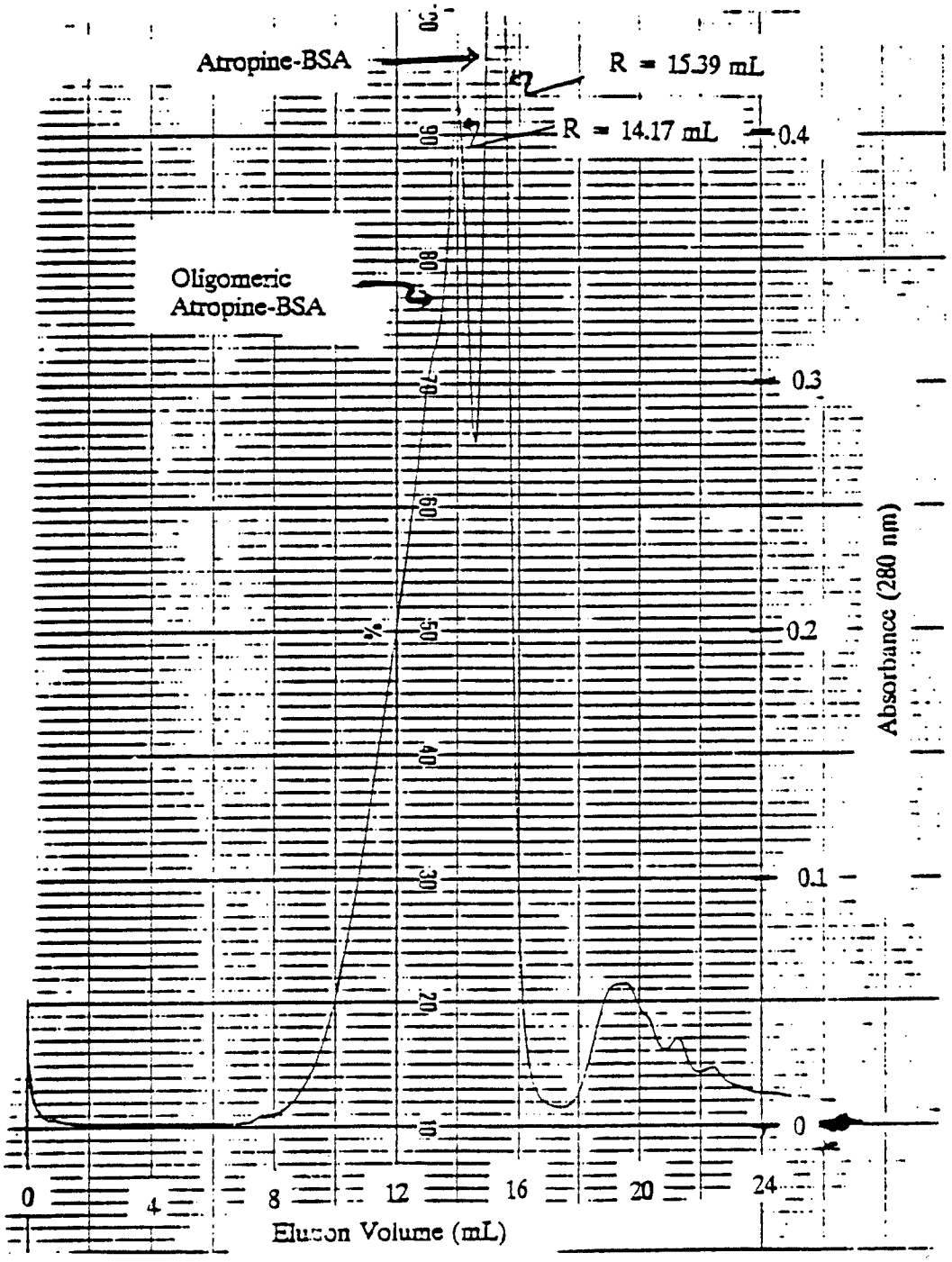


FIGURE 5. SIZE EXCLUSION CHROMATOGRAPHY OF ATROPINE-BSA CONJUGATE. 200 μL ATROPINE-BSA (1.0 mg/mL) WAS APPLIED TO A SUPEROSE-6 COLUMN

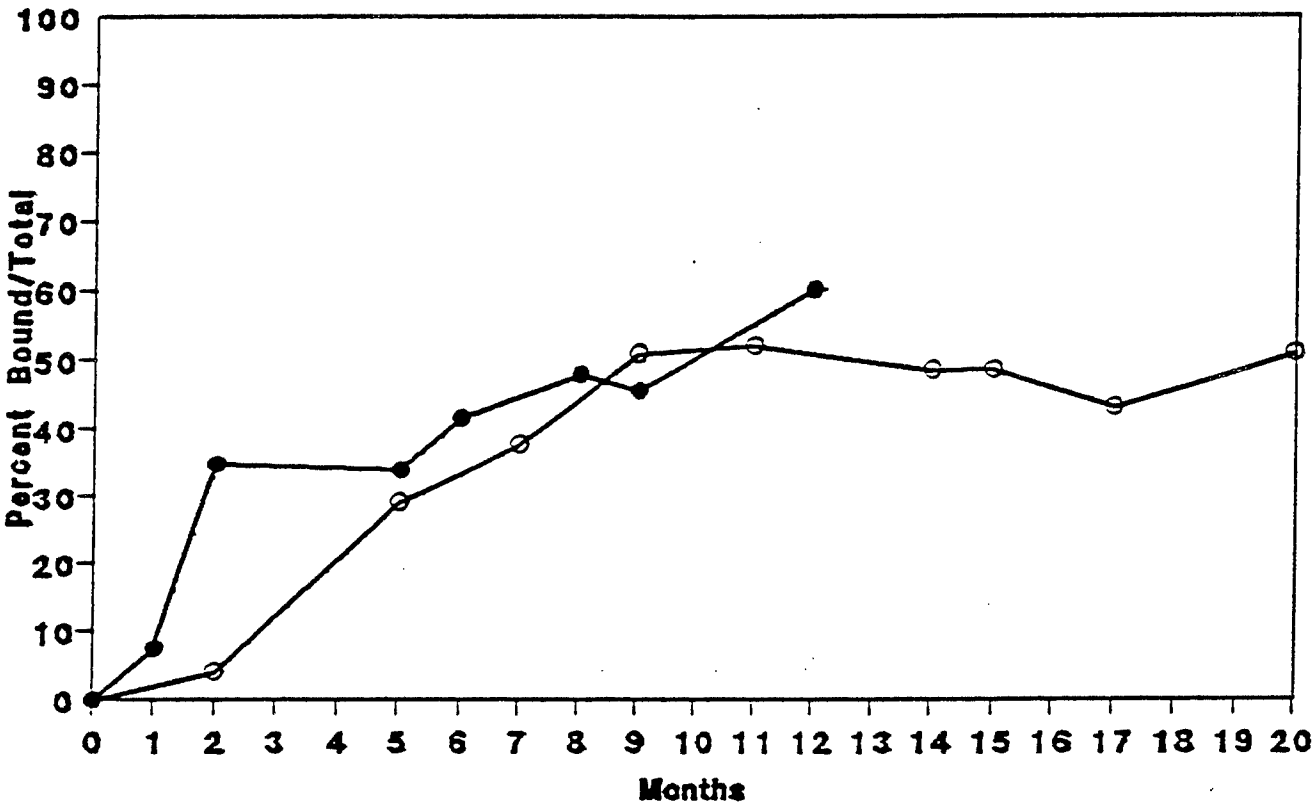


FIGURE 6. DEVELOPMENT OF ANTI-ATROPINE ANTIBODY RESPONSE. TITER IS EXPRESSED AT %B/T FOR 1:100 SERUM DILUTIONS. -o- RABBIT E6345; -●- RABBIT 33088.

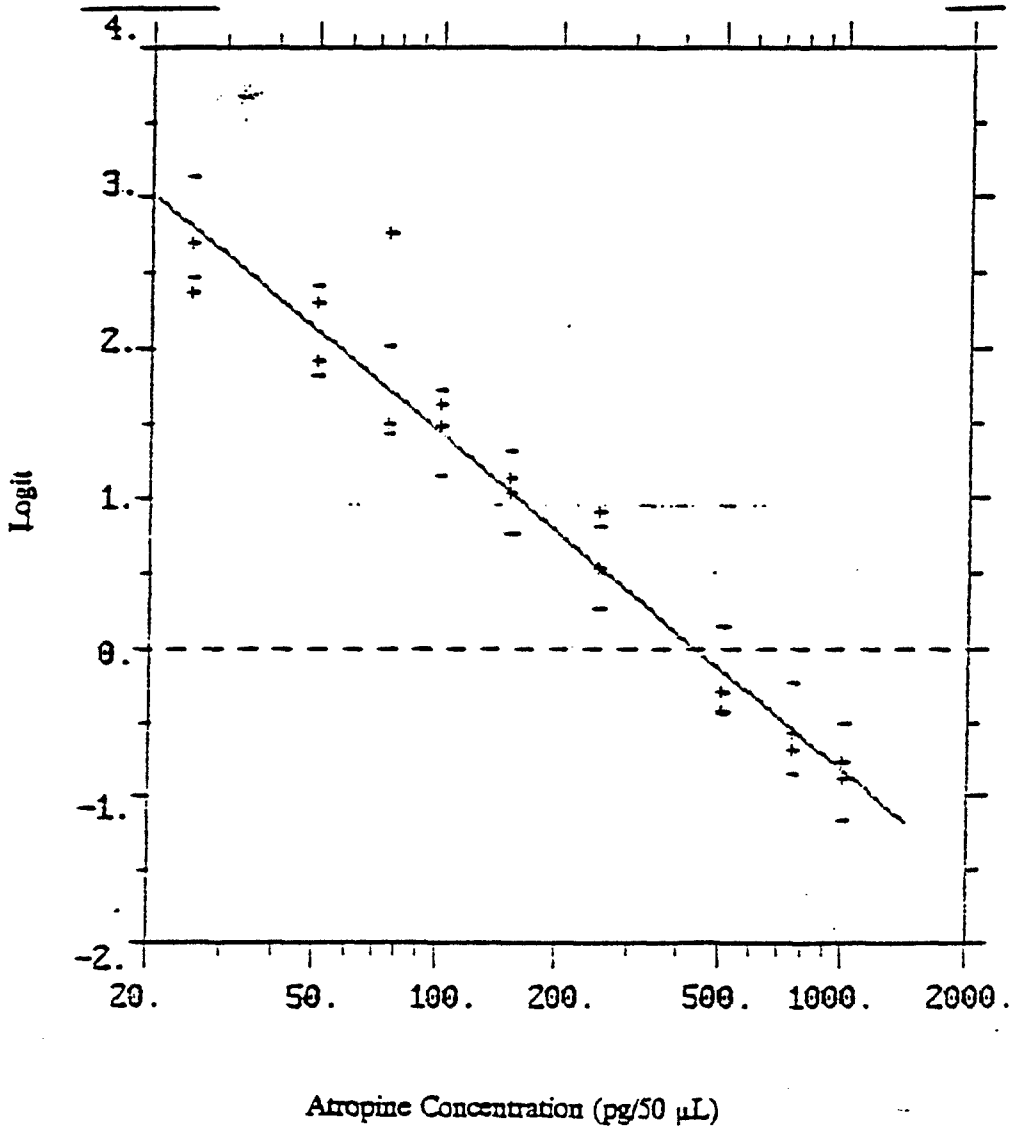


FIGURE 7. ATROPINE STANDARD CURVE FOR POOLED ANTI-ATROPINE ANTIBODY
The antisera was diluted 1:600 and the plot is an unweighted linear regression.

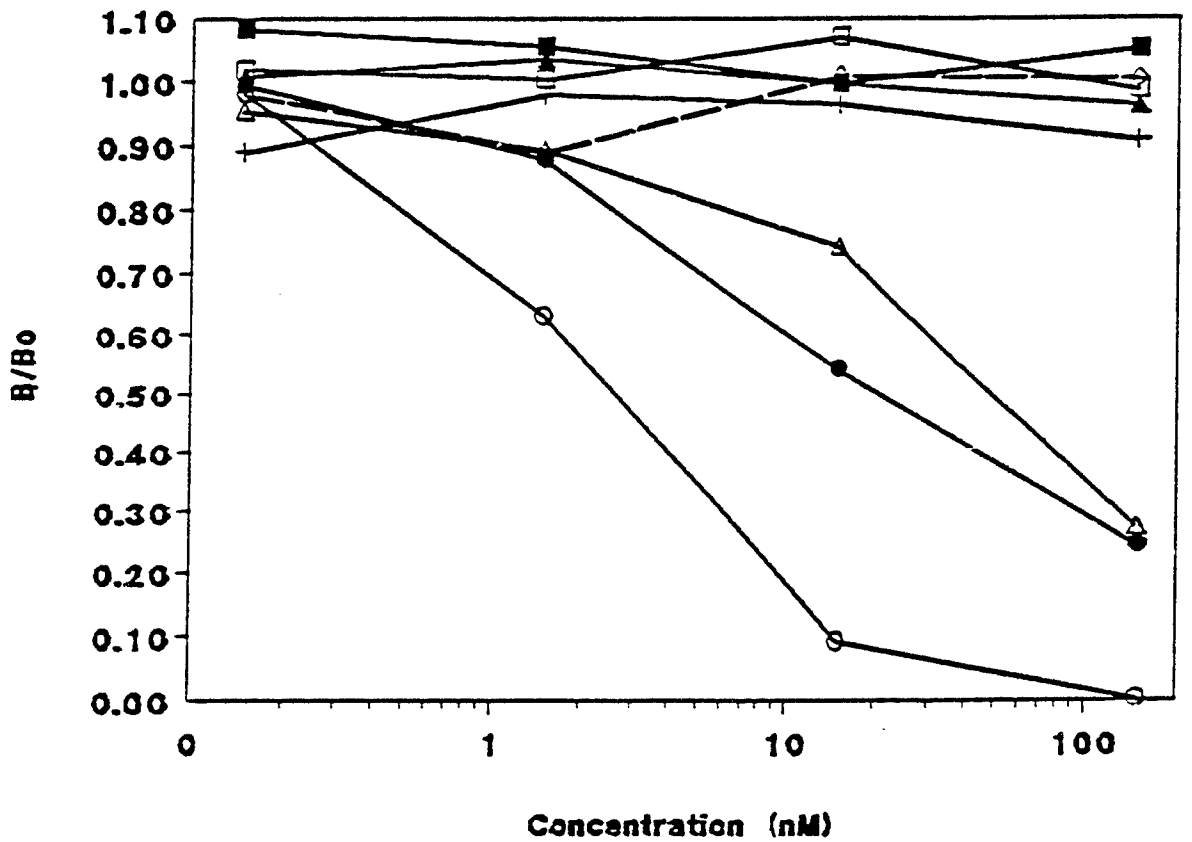


FIGURE 8. SPECIFICITY OF ANTI-ATROPINE ANTIBODY.
-o-, ATROPINE; -•- DL-HOMATROPINE;
-Δ- L-HYOSCYAMINE; -▲- (-) SCOPOLAMINE;
-|- ATROPINE METHYL NITRATE; -□- ACETYLCHOLINE IODIDE; -■-D,L-TROPIC ACID; - - TROPINE

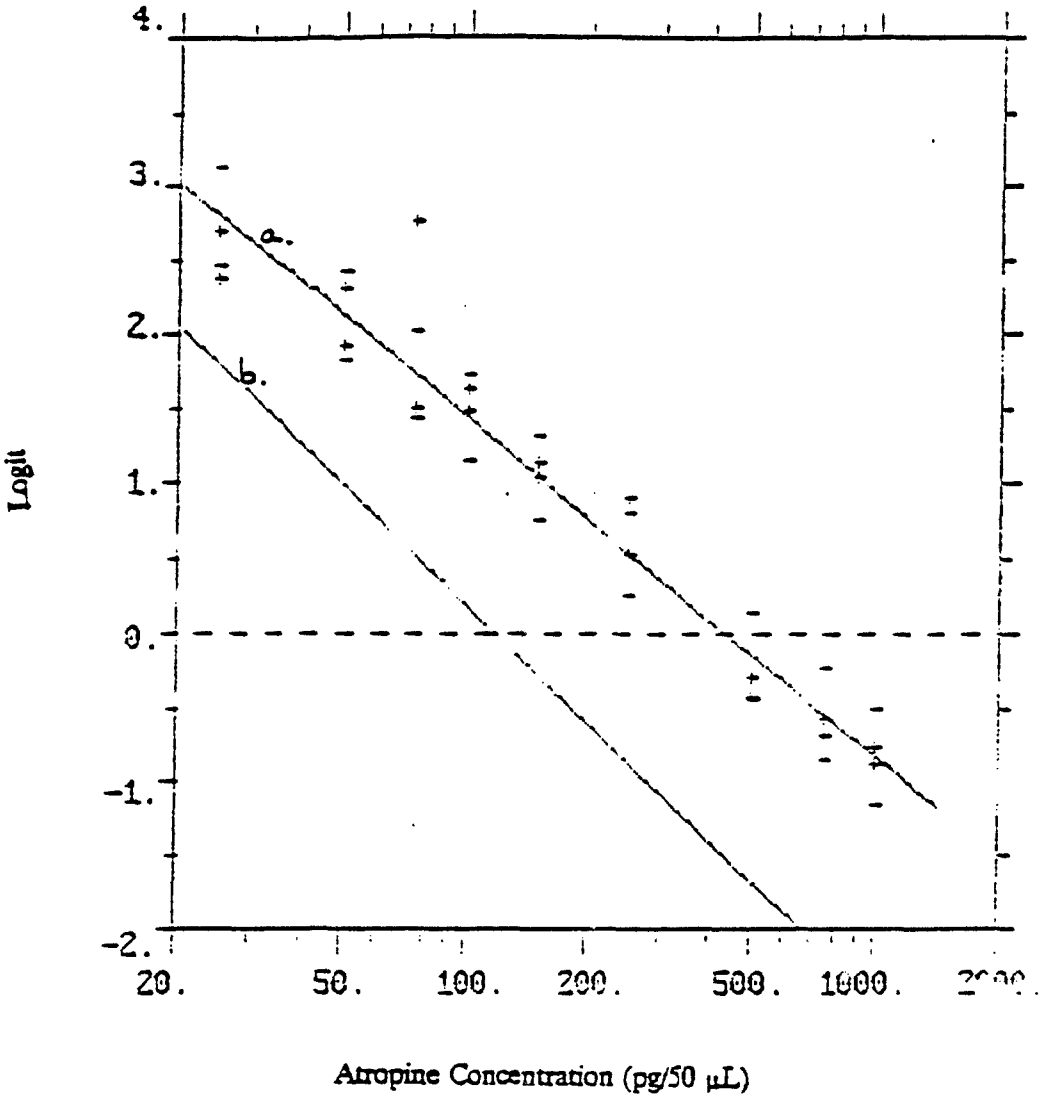


FIGURE 9. COMPARISON OF BATTELLE AND WRAIR ANTISERA. Anti-atropine antisera was diluted to 1:600 for Battelle (a) and 1:1600 for WRAIR (b).

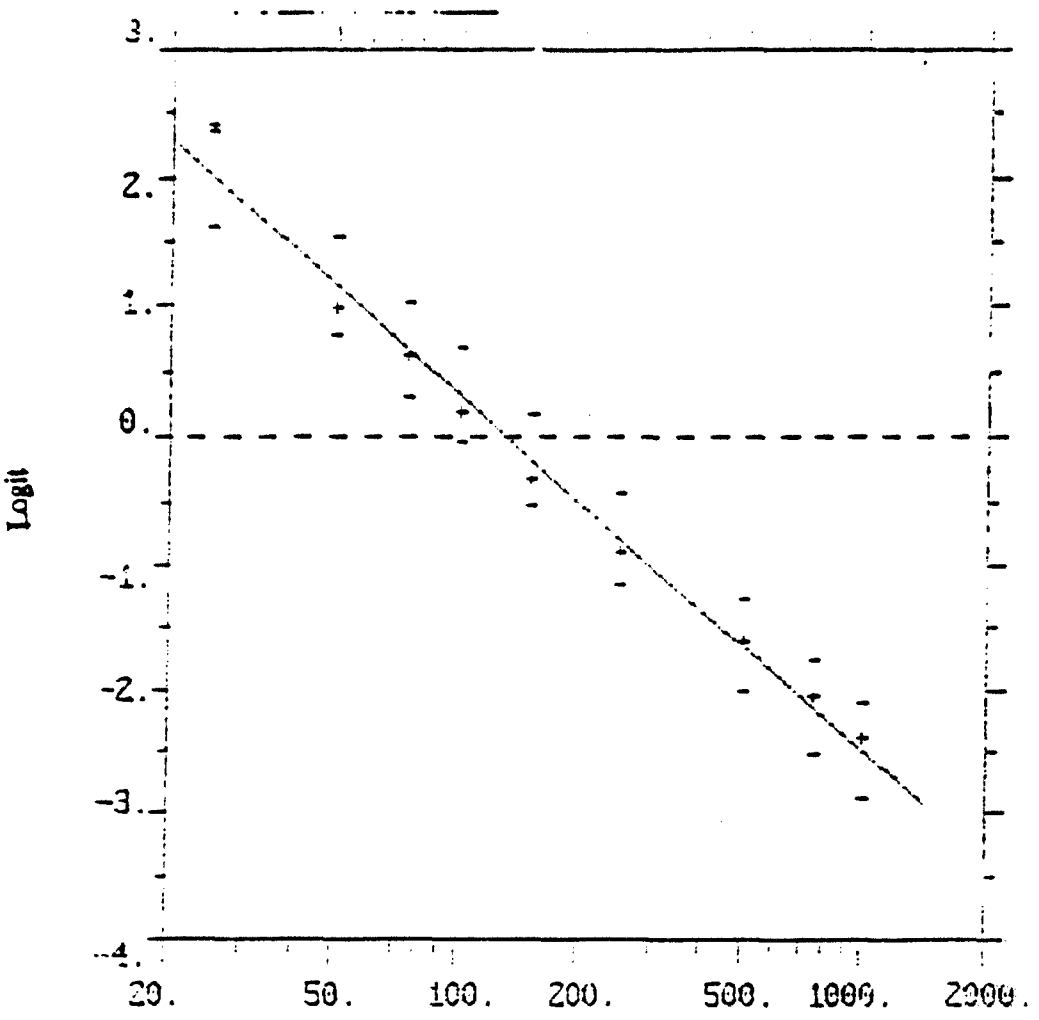


FIGURE 10. ATROPINE STANDARD CURVE. THE CURVE WAS GENERATED MANUALLY USING WRAIR ANTISERA DILUTED 1:1600 DURING THE VALIDATION PHASE OF THE STUDY.

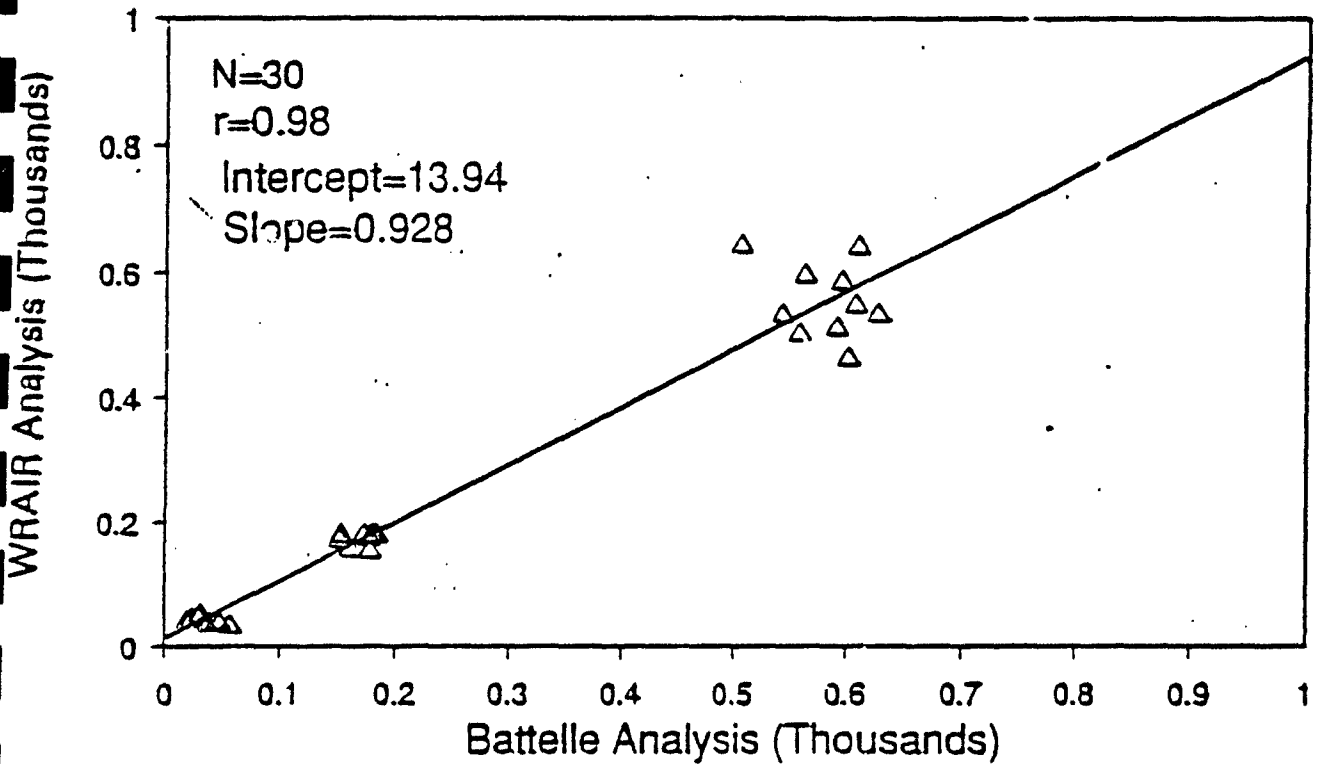


FIGURE 11. INTERLABORATORY COMPARISON OF ATROPINE RIA SAMPLES PREPARED AT WRAIR

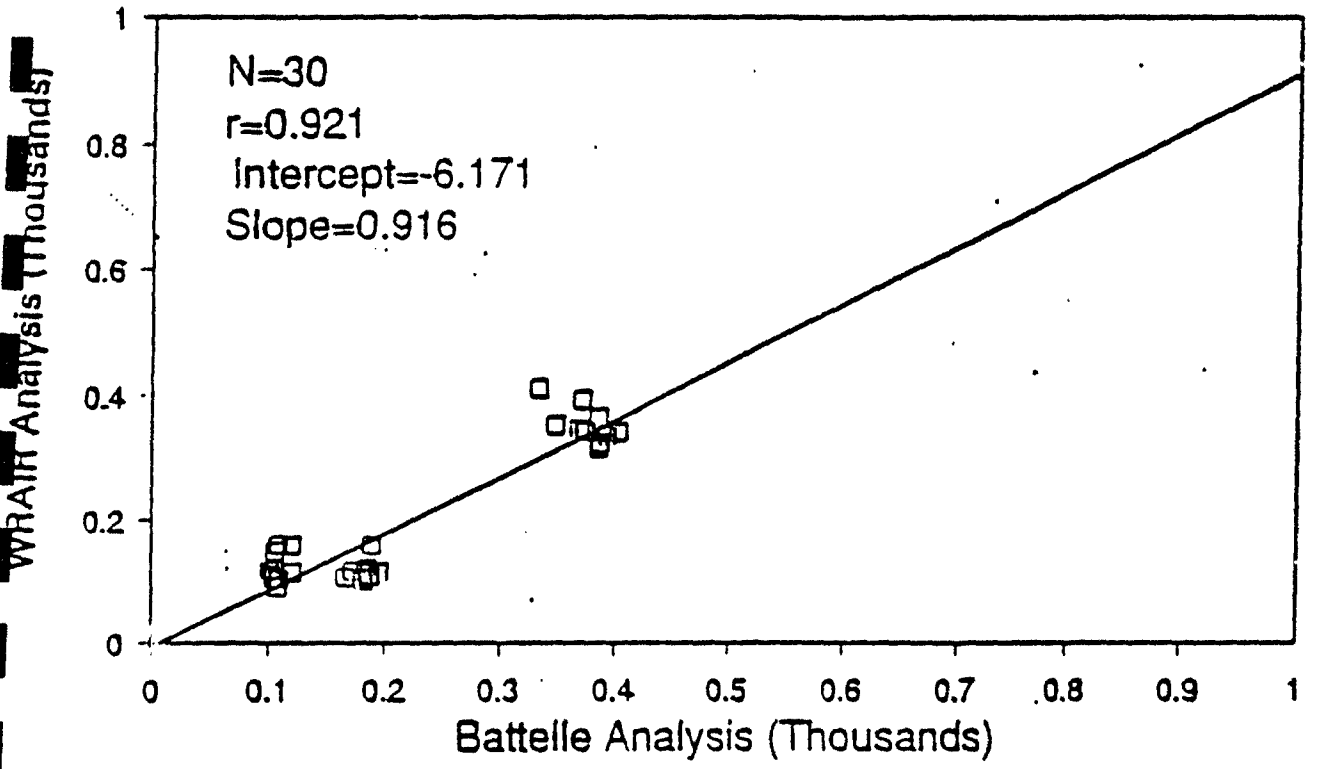


FIGURE 12. INTERLABORATORY COMPARISON OF ATROPINE RIA SAMPLES PREPARED AT BATTELLE

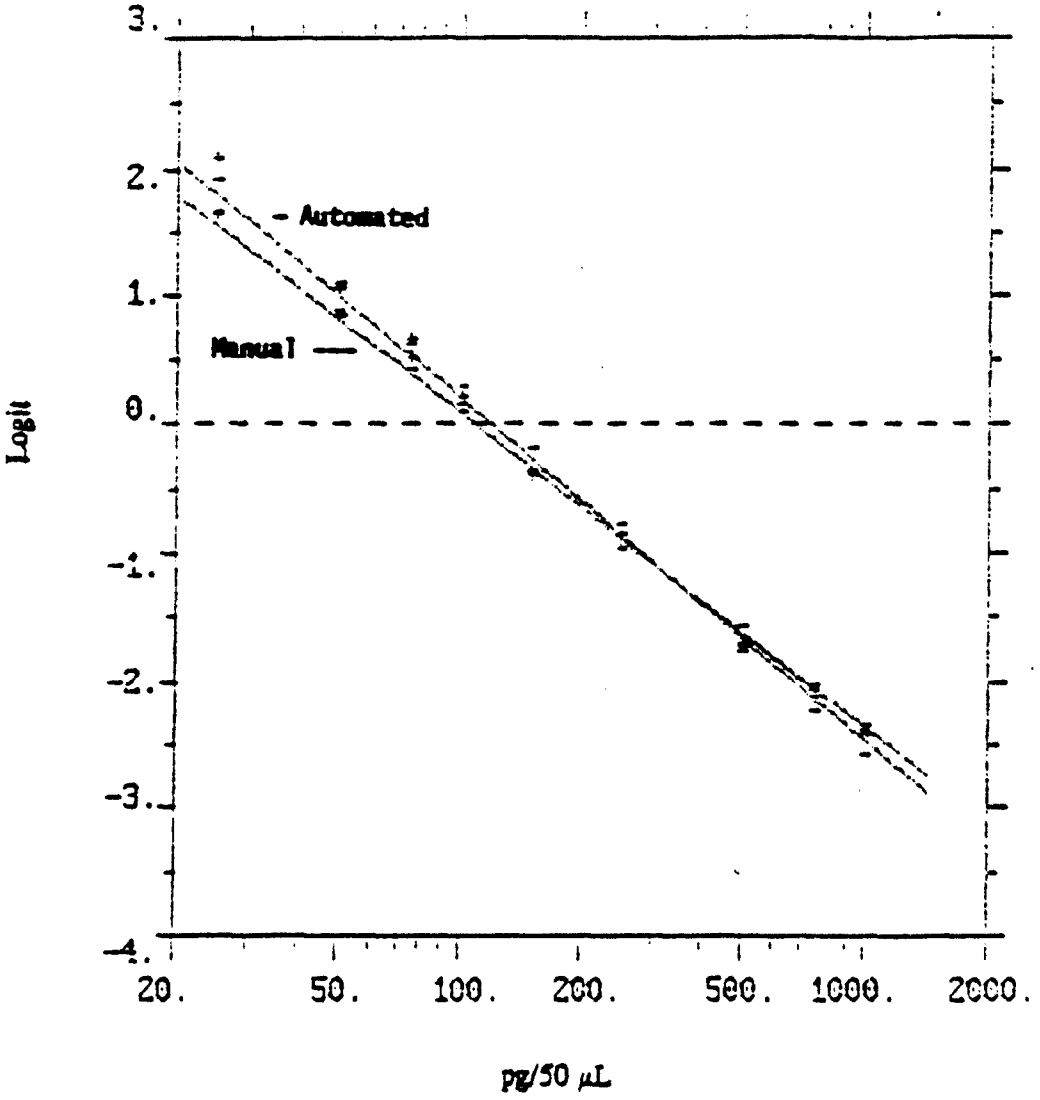


FIGURE 13. COMPARISON OF STANDARD CURVES FOR MANUAL AND AUTOMATED ATROPINE RIAS

APPENDIX D

TABLES

TABLE 1. UV ABSORPTION OF ATROPINE, BSA, AND ATROPINE-BSA CONJUGATE

Sample	Absorption Bands[a]					
	1		2		3	
	λ_{max}	$E^{0.01\%}_{1\,cm}$	λ_{max}	$E^{0.01\%}_{1\,cm}$	λ_{max}	$E^{0.01\%}_{1\,cm}$
BSA	278 nm	0.066				
Atropine Sulfate	250 nm	0.44	256 nm	0.53	262 nm	0.41
Atropine-BSA	268 nm	1.080	340 nm	0.520		

[a] Determined on Beckman DU-50 Spectrophotometer.

TABLE 2. SUMMARY OF TNBS ANALYSIS FOR BSA AND ATROPINE-BSA

Sample	nmoles BSA/mL	A_{335}	nmoles Amino* Groups/mL	molar ratio (amino groups:BSA)
BSA-50 µL	0.65	0.246	24.6	37.8
BSA-100 µL	1.30	0.535	53.5	41.2
Atr-BSA-50 µL	0.746	0.055	5.5	7.4
Atr-BSA-100 µL	1.492	0.103	10.3	6.9

* nanomoles of free amino groups/mL = corrected A_{335} x 100.

TABLE 3. RABBIT ANTI-ATROPINE TITERS

Group	Rabbit Number	% Bound[a]
I	E6345	44
	E6370	32
2	33088	45
	32564	10
	32492	12
	33169	5
	32578	15
	32522	20
	32597	26
	33023	32

[a] % binding at 1:100 dilution

TABLE 4. SPECIFICITY OF ANTI-ATROPINE ANTISERA

Compound	ID_{50} (nM)	% Crossreactivity
atropine sulfate	2.11	-
l-hyoscyamine	60.58	3.5[a]
d,l-homatropine	22.49	9.4
scopolamine	≥148	≤1.4
acetylcholine iodide	≥148	≤1.4
atropine methyl nitrate	≥148	≤1.4
tropine	≥148	≤1.4
tropic acid	≥148	≤1.4

[a] $\%\ Cross\ Reactivity = \frac{ID_{50}\ Atropine\ Sulfate}{ID_{50}\ Test\ Compound} \times 100\%$

TABLE 5. COMPARISON OF BATTELLE AND WRAIR ANTISERA

Parameter	Battelle	WRAIR
Initial Dilution	1:600	1:1600
R/T	0.32	0.57
B/T	0.01	0.01
Slope	0.984	1.156
y-intercept	5.975	5.515
correlation coefficient	0.99034	0.99917
ED_{20}	1770	392.5
ED_{50}	432.7	118.3
ED_{80}	105.8	35.63
Detection Limit	3.0 ng/mL	1.5 ng/mL
Response Range	3.0 - 20.0 ng/mL	1.5 - 20.0 ng/mL
Binding Constant	9.75×10^{-11} M^{-1}	2.8×10^{-10} M^{-1}

TABLE 6. STATISTICAL ANALYSIS OF STANDARDS FOR ATROPINE RIA INHIBITION CURVE

Calibration Point (Atropine, pg)	N	Mean Counts	Std. Dev.	% CV	B/B_o
NSB	4	68.9	± 2.97	4.3	--
Reference (Zero)	14	6779.7	± 207.7	3.1	100
25 pg	2	6202.1	± 139.3	2.3	91.4
50 pg	10	4956.3	± 385.4	7.8	72.8
75 pg	2	4436.4	± 44.1	1.0	65.1
100 pg	2	3754.7	± 298.7	7.9	54.9
150 pg	11	2879.5	± 117.5	4.1	41.9
250 pg	2	2022.8	± 15.5	0.8	29.1
500 pg	12	1189.7	± 40.96	3.4	16.7
750 pg	2	840.5	± 16.83	2.0	11.5
1000 pg	12	632.3	± 33.13	5.2	8.4
2000 pg	1	310.9	---	---	4.6

TABLE 7. RECOVERY STUDIES OF ATROPINE IN RHESUS MONKEY SERUM

Atropine (in Serum)	N	Sample Size (µL)	Expected Level (pg/50 µL)	Measured Level (pg/50 µL)	Percent Recovered
2 ng/mL	5	50	100 pg	99 ± 2.1	99 ± 2.1
10 ng/mL	5	50	500 pg	528 ± 11	105.6 ± 2.2
20 ng/mL	5	50	1000 pg	920 ± 57	92 ± 5.7

TABLE 8. COMPARISON OF RECOVERY STUDIES FOR ATROPINE IN SPIKED RHESUS MONKEY SERA PREPARED AT WRAIR AND ANALYZED AT BATTELLE

Actual Concentration (pg/50 μL)[a]	Measured Concentration (pg/50 μL)[b]	% Expected[d]
0	≤25	100
50	38.4 ± 10.7[c]	77
150	171.3 ± 11.4	114
500	580.2 ± 36.9	116

[a] The actual concentration of atropine in the sample as prepared by WRAIR.

[b] The atropine concentration in the sample as determined at Battelle during the interlaboratory comparison.

[c] Mean ± standard deviation of duplicate determinations.

[d] % expected calculated by dividing measured concentration by actual concentration.

TABLE 9. OUTLINE FOR THE MANUAL AND AUTOMATED ATROPINE RIA

Assay Step	Time (minutes)			
ASSAY	Manual		Automated	
Set up	Attended	Elapsed	Attended	Elapsed
prepare reagents*	60	60	60	60
set up test tubes in racks*	60	60	60	60
Assay				
add reagents (7) to tube**	300	300	5	45
vortex each tube	30	30	30	30
incubate 20 hours	5	1200	5	1200
clean up	30	30	30	30
add saturated ammonium sulfate	45	45	45	45
vortex	15	15	15	15
incubate 15 minutes	0	15	0	15
centrifuge	10	30	10	30
aspirate supernatant	60	60	60	60
add 50% satd ammonium sulfate	45	45	45	45
vortex	15	15	15	15
incubate 15 minutes	0	15	0	15
centrifuge*	10	30	10	30
aspirate	60	60	60	60
add dH20	45	45	45	45
vortex	15	15	15	15
transfer to scintillation vial	60	60	60	60
add LSC solution*	60	60	60	60
DETECTION				
transfer vials to counter*	30	30	30	30
set up LS counter/computer	5	5	5	5
count vials (approximately 10 min/tube)	0	1400	0	1400
clean up	30	30	30	30
DATA ANALYSIS				
set up RIA/CALC software	5	5	5	5
manually enter data	30	30	30	30
analyze data	5	5	5	5
review data	30	30	30	30
document data	180	180	180	180
TOTAL	1240	3905	945	3650

* Step performed by second technician.
Time in minutes for 140 tubes (standards, 40 samples, and controls in duplicates)

** Automated steps performed by Tecan liquid handling system

TABLE 10. COMPARISON OF THE MANUAL AND AUTOMATED ATROPINE RIA[a]

Parameter	Manual	Automated
R/T	0.67	0.57
B/T	0.01	0.01
Slope	1.135	1.156
y-intercept	5.388	5.515
correlation coefficient	0.99835	0.99917
ED_{20}	392.1	392.5
ED_{50}	115.5	118.3
ED_{80}	34.04	35.63
Detection Limit	0.5 ng/mL	0.5 ng/mL
Response Range	0.5-20.0 ng/mL	0.5-20.0 ng/mL
% CV for Standards	0.24%-7.63%	1.01%-5.05%

[a] Manual and automated assays performed with WRAIR antisera diluted 1:1600

TABLE 11. COMPARISON OF THE PRECISION OF MANUAL AND AUTOMATED ATROPINE RIAS

	Automated			Manual		
Standard (pg/50 µL)	Mean CPM	Std. Dev.	% CV	Mean CPM	Std. Dev.	% CV
NSB	46.3	3.6	7.8	50.3	3.3	6.61
Reference (0)	2768.7	28.0	1.01	3127.0	109.5	3.50
25	2399.1	99.2	4.13	2362.2	5.7	0.24
50	2017.8	79.5	3.94	2392.6	182.6	7.63
75	1797.3	64.1	3.56	2042.2	42.1	2.06
100	1537.8	26.9	1.75	1726.3	19.6	1.13
150	1150.6	13.8	1.20	1288.7	38.4	2.98
250	832.0	42.0	5.05	901.5	27.5	3.05
500	462.5	4.9	1.07	529.1	9.2	1.73
750	344.8	7.8	2.26	386.9	7.2	1.86
1000	271.8	7.0	2.58	309.4	5.8	1.87